Ecotoxicity of nanomaterials in relation to the freshwater blackworm, *Lumbriculus variegatus*

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Abstract

Nanotechnologies are increasing being applied within consumer products, science, medicine, engineering and industrial processes. Due to their small size and high reactivity, nanoparticles (NPs) possess a potentially unique risk towards humans and the environment. Although the aquatic environment is predicted to be the final recipient of nano-wastes, little is known regarding the potential toxicity of NPs towards aquatic species. Within this thesis, the toxicity of two reference nanoparticles: silver (NM-300K) and titanium dioxide (NM-104) were investigated in relation to the freshwater oligochaete, *Lumbriculus variegatus* in artificial water and sediment. Greater toxicity was observed for NM-300K towards *L. variegatus* in both short-term (96-hour) water and long-term (28-days) sediment exposures. Sub-lethal NM-300K aquatic exposures (0.2 mg/L) led to an inhibition of *L. variegatus* swimming behaviours designed for the escape of predation as well as increases in the antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT). Increases in SOD and CAT indicate the onset of oxidative stress, however, no oxidative damage (as measured by lipid peroxidation) was observed. The inclusion of Suwannee River humic acid (5mg/L) as a natural organic matter (NOM) source generally reduced NM-300K toxicity in aquatic exposures – improving swimming behaviours and limiting SOD and CAT activities to those recorded in control exposures. Although NM-300K displayed greater toxicity than NM-104 in sediment exposures, 100% *L. variegatus* mortality was only observed at extremely high, potentially environmentally irrelevant concentrations (1333.33 mg/kg). The results of this thesis demonstrate the importance of water and sediment NP toxicity testing in relation to *L. variegatus* using traditional and newly emerging endpoints and variations to abiotic conditions.

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List of abbreviations

AAS	Atomic Adsorption Spectroscopy
AFM	Atomic Force Microscopy
AgNP	Silver nanoparticle
ANOVA	Analysis of variance (one-way)
BSA	Bovine serum albumin
CAT	Catalase
CNT	Carbon nanotubes
CSM	Colorado School of Mines
DCS	Differential centrifugal sedimentation
DDT	Dichlorodiphenyltrichloroethane
dH_2O	Distilled water
DTPA	Diethylene triamine pentaacetic acid
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic acid
DVLO	Derjaguin-Landau-Verwey-Overbeek
d.w	Dry weight
EDL	Electrical double layer
EDS	Energy dispersive x-ray spectroscopy
EDTA	Ethylenediaminetetraacetic acid
ENM	Engineered nanomaterial
EPA	Environmental Protection Agency

EPSRC	Engineering and Physical Sciences Research Council
FeSOD	Iron superoxide dismutase
FFF-ICP-MS	Field-Flow Fractionation Inductively Coupled Mass Spectroscopy
GLM	General linear model
GPX	Glutathione peroxidase
GSH	Glutathione
GST	Glutathione-S-Transferase
H_2O_2	Hydrogen peroxide
$HO^{\bullet}$	Hydroxyl radical
HA	Humic acid
HD	Hydrodynamic diameter
HWU	Heriot Watt University
ICP-MS	Inductively Coupled Plasma Mass Spectroscopy
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectroscopy
ISE	Ion Selective Electrode
JRC	Joint Research Centre
$L^{\bullet}$	Fatty alkyl free radical
LENNF	Leeds EPSRC Nanoscience and Nanotechnology Facility
LGF	Lateral giant fibres
$LOO^{\bullet}$	Fatty peroxyl radical
$LOOH^{\bullet}$	Lipid hydroperoxide
MARINA	Managing Risks of Nanomaterials
MDA	Malondialdehyde

MGF	Medial giant fibres
MnSOD	Manganese superoxide dismutase
MOPS	3-(N-Morpholino)propanesulfonic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NM	Nanomaterial
NP	Nanoparticle
NOM	Natural organic matter
$O_2^{\bullet-}$	Superoxide anion
OECD	Organisation for Economic Co-operation and Development
OD	Optical density
PAHs	Polycyclic aromatic hydrogen
PBS	Phosphate buffered saline
PCBs	Polychlorinated biphenyls
PdI	Polydispersity index
PEC	Predicted environmental concentration
PENC	Predicted no effect concentration
PUFA	Polyunsaturated fatty acid
PVP	Polyvinylpyrrolidone
PZC	Point of zero charge
QSAR	Quantitative structure-activity relationship
ROS	Reactive oxygen species
SD	Standard deviation
SP-ICP-MS	Single-particle Inductively Coupled Mass Spectroscopy

SPSS	Statistical Package for the Social Sciences
SOD	Superoxide dismutase
SRHA	Suwannee River humic acid
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TCA	Trichloroacetic acid
TEM	Transmission Electron Microscope
TiO_2NP	Titanium dioxide nanoparticle
TMAH	Tetramethylammonium hydroxide
UV	Ultraviolet
ZP	Zeta poten

Chapter 1: Introduction

1.1 Nanomaterial and nanoparticle definitions

The EU recommended (2011/696/EU) definition of a nanomaterial is as follows:

A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm.

Nanoparticles, a sub-set of nanomaterials are defined as particles with two or three external dimensions within the nano range (British Standards Institution, 2007; ASTM, 2012). Due to their small size, nanoparticles (NPs) possess several unique characteristics, (e.g. optical, electronic, electromagnetic, thermodynamic and chemical features) which are not exhibited in their bulk form counterparts. In addition, they are typically more reactive due to a larger surface area to volume ratio. NPs can occur naturally through biogenic, geogenic, pyrogenic and atmospheric processes or anthropogenically, either as a by-product of combustion (e.g. coal) or via their deliberate manufacturing (Nowack and Bucheli, 2007). Deliberately produced materials with one or more dimension within the nanoscale are defined as engineered nanomaterials (ENMs)

Due to their large surface energy, NPs tend to cluster to form strongly bound aggregates or weakly bounded agglomerates. The total surface areas of aggregates are significantly smaller than the combined surface area of the primary particles whereas agglomerate surface areas are generally considered to be a similar size (ISO, 2011). Despite potential changes in surface area, aggregates and agglomerates may still exhibit the same properties as unbound NPs. Furthermore, NPs may be released from aggregates or agglomerates throughout their life cycle. Consequently, the definition of nanomaterial is still applicable to NPs in an aggregated or agglomerated state (EU, 2011).

1.2 Nanotechnology

Through the control of nanoscale materials and the exploitation of their unique physicochemical properties, the rapidly expanding field of nanotechnology has the potential to revolutionise science, industry and society as a whole. The concept of nanotechnology was first introduced by Richard Feynman in his 1959 lecture "There's plenty of room at the bottom" whilst the term itself was later defined by Professor Norio Taniguchi, Tokyo Science University in 1974 as the "processing of, separation, consolidation, and deformation of materials by one atom or one molecule". The emergence of the scanning tunnel microscope capable of observing and controlling nanoscale particles coupled with the development of cluster science significantly enhanced the research and development of nanotechnology in the early 1980's (Aguilar, 2013). The global prioritisation of nanoscale science throughout the 1990's and 2000's has led to forecasts of nanotechnology becoming a 'general-purpose' technology by 2020 (Roco, 2011). Nanotechnology is currently used in numerous sectors, including (but not limited to), pharmaceuticals; cosmetics; textiles; chemicals; electronics; food and environmental remediation.

1.3 Nanoparticles in the environment

Given the exponential rate at which nanotechnology has grown over the past three decades, human and environmental exposure to NPs will inevitably increase. The unintentional release of nano-waste may occur during the synthesis of raw nanomaterials, the production and transportation of nano-products, their use in consumer products and their eventual disposal. Whilst the potentially advantageous implications of nanotechnology are overwhelming, understanding the possible effects of NP exposure on human health and the environment must also be considered a priority. Due to their small size, unique physicochemical characteristics and high reactivity, NPs represent a risk unlike any bulk materials. By developing protocols for the testing of NPs, the field of nanotoxicology (a subcategory of toxicology) aims to address knowledge gaps in relation to their potential adverse effects upon human health (Donaldson *et al,* 2004). Nanotoxicological research has revealed NPs to induce

oxidative stress and pro-inflammatory effects on the lung which can lead to genotoxicity and cytotoxicity (Nel *et al,* 2006).

Given these findings, it can be assumed that upon their release into the environment, NPs may also exhibit adverse effects. Consequently, the field of ecotoxicology (a relatively new science concerned with the contamination of the biosphere and the effect of contaminants upon its constituents) has also embraced the study of the potential environmental hazards posed by NPs via nanoecotoxicology. Whilst nanotoxicological research began in the early 1990's, nanoecotoxicology is deemed to have begun in the mid 2000's (Kahru and Dubourguier, 2010) and is still in its infancy.

The unique composition, structure and size of NPs will influence the route and rate at which they enter the environment. Similarly, it will control how they behave once released in the environment, in terms of; biological uptake, how their behaviour is influenced by different abiotic factors (natural organic matter, pH and ionic strength) and whether they interact with other pollutants. Handy *et al.* (2008) hypothesised that NPs could potentially reduce the bioavailability of other, non-nano pollutants through adsorption, and that under some circumstances they could also act as a delivery vehicle. The heterogeneous and transient nature of the environment also make it challenging to anticipate how NPs will behave upon their release, however, NPs are predicted to be highly mobile within porous media (e.g. groundwater aquifers) (Lecoanet *et al,* 2004) and have the potential to be transported long distances. With this in mind, it is possible that NPs could be easily transported from an area where they are relatively inert to an area where they can be altered or transformed and subsequently exert toxicity.

Aquatic environments are well documented recipients of industrial and commercial wastes and despite preventative measures, will inevitably receive an influx of nanowastes in the near future (Nowack & Bucheli, 2007). However, quantifying the concentration of NPs in wastewaters and the environment is challenging due to a lack of cost effective, sensitive analytic techniques. Whilst relevant analytic tools are being developed, current estimates of environmental NP concentrations are based on forecasted values of NP production, the life cycle of nano-products and environmental fate models. By studying the physicochemical properties of NPs and how they interact with the environment and environmental processes; environmental fate models have allowed estimates of NP fate in various environmental components to be made

(Praetorius *et al,* 2012). To date a number of environmental fate models have been used to generate predicted environmental concentrations (PEC) and/or predicted no effect concentrations (PNEC) for the most commonly used NPs such as silver, titanium dioxide and zinc oxide in water, soil and air (Mueller and Nowack, 2008; Blaser *et al,* 2008; Gottshalk *et al,* 2009).

Environmental fate models are used to shape the risk assessment of potentially hazardous materials, however, they are still very much in their infancy in regards to NPs. With the overall aim of developing nano-relevant risk assessments, the European Commission has highlighted the need for research which assesses the aquatic toxicity, degradability and bioaccumulation of NPs. The NPs commonly tested (but not exclusively) in nanotoxicology investigations consist of those outlined by the Organisation for Economic Co-operation and Development (2010) as nanomaterials anticipated to experience an increase in the nano-market share, including: aluminium oxide, carbon black and nanotubes, cerium oxide, fullerenes, iron oxide, silicon oxide, silver, titanium and zinc oxide.

1.4 Silver

1.4.1 Occurrence and applications of silver

Silver is a rare, yet naturally occurring precious metal - the Earth's crust is estimated to comprise only 0.07 mg/kg silver, the majority of which is found in basalt and igneous rocks (Nadkarni and Morrison, 1975). Silver is most often present as a mineral in association with other elements, such as iron, copper, gold and lead (Panyala, 2008). Due to its well renowned antibacterial properties, silver has a long association with medical applications which date back to ancient civilisations. Metallic silver was used in plates designed to heal wounds and fight against surgical infections, whilst silver nitrate and various other salts of silver have been used in medicine for the treatment of; burns (Klasen, 2000), ulcers (Alexander, 2009; Klason, 2000), brain infections (Alexander, 2009), fistulae (Landsdown, 2002; Sims, 1884), ophthalmia neonatorium (in eye drops) (Schneider, 1984) venereal diseases (Klasen, 2000) and as wound dressings (Chopra, 2007; Demling and DeSanti, 2001). Antibacterial properties of silver are also employed

in water purification applications to rid drinking water of bacteria and algae (Davies and Etris, 1997).

In its pure form, silver has the highest electrical and thermal conductivity of all metals, whilst having the lowest contact resistance, which has made it a highly desirable material across almost all sectors of industry. The incorporation of silver to electrical and motor control switches is universal as it is non-corrodible, whilst it is one of only a few elements which can optimise chemical reactions used to develop a number of commercial products (Findik and Uzun, 2003). The reflective capabilities of silver have seen it become incorporated into windshields (Wolfe and Young, 1990), mirrors, and coatings as well as jewellery, where its malleability is also advantageous (Johnson *et al,* 2005). Although other technologies are now available, the high photosensitivity of silver halide still makes silver-based photography popular due to its high definition and low costs (Bergthaller, 1996). The highly conductive nature of silver is exploited within solar panels to collect electrons produced by sunlight and ultimately provide an alternative energy source.

1.4.2 Silver health effects

Silver has no known physiological role in the human body and is thus considered a toxic heavy metal. Adverse health effects associated with silver exposure include; the irreversible blue/grey pigmentation of skin (argyria) or eyes (agyrosis) (ATSDR, 1990), respiratory irritation (Rosenman *et al,* 1979), bronchitis, emphysema and reduced red blood cell function (Baldi *et al,* 1988). Silver nanoparticles (AgNPs) are also reported to have adverse implications on human health. Several *in vitro* studies using mammalian cells have indicated the toxicity of AgNP towards the; skin (Larese *et al,* 2009), lungs (Foldbjerg *et al,* 2011), liver (Piao *et al,* 2011), reproductive organs (Asare *et al,* 2011) and brain (Trickler *et al,* 2010). *In vivo,* AgNPs have demonstrated pulmonary retention and tissue distribution (e.g. to the liver) following pulmonary exposure (Takenaka *et al*, (2001), inflammatory responses, alterations to lung function (Sung *et al,* 2009) and neurotoxicity (Rahman *et al,* 2009). Furthermore, AgNPs may also exhibit toxicity at non-cytotoxic doses, for example, in human hepatoma cells where cell proliferation and the induction of genes associated with cell-cycle progression were observed following

exposure to AgNP (Kawata *et al,* 2009). Sub lethal AgNP doses are also known to induce inflammation and oxidative stress within human cells (Lim *et al,* 2012).

1.4.3 Silver in the environment

The discharge of silver from numerous industrial applications into the environment is well documented and although incidents of silver induced environmental toxicity are relatively rare (Rodgers *et al,* 1997), it is thought to have some ecological effect. Laboratory testing indicates that ionic or free silver is one of the most toxic metals to aquatic organisms; including fish (Wood *et al,* 1999), algae (Hiriart-Baer *et al,* 2006) and *Daphnia magna* (Bianchini and Wood, 2009). However, ionic silver is readily tranformed to less innocuous forms upon its interaction with natural chemical ligands (i.e. in sewer systems, treatment facilities and within the environment) and is consequently less toxic (Purcell and Peters, 2009).

Given its low concentration in the Earth's crust, and low mobility background levels of silver in natural waters are low. Consequently, small anthropogenic releases of silver may cause large deviations from normal levels (Luoma, 2008). Concentrations of silver are typically within the part per trillion (or ng/L) range, however data regarding freshwater concentrations of silver are scarce and can vary per location (i.e. concentrations in urban effluents are considerably higher than those in natural waters). Wen *et al* (2002) found silver concentrations from city effluents to range from 64-327 ng/L, whilst concentrations in natural aquatic bodies have been recorded at 10 ng/L in pristine conditions and between 10-100 ng/L in urbanised areas (Ratte, 1999)

1.4.4 Applications of AgNPs

Given the popularity of silver, it is unsurprising that AgNPs have emerged as the most commonly used NPs in consumer goods (Rejeski, 2009) in addition to a plethora of industrial applications. The Woodrow Wilson inventory on nanotechnology-based consumer products currently (August, 2016) lists 442 commercially available products containing silver nanoparticles, including; clothing, sports equipment, inks, soap,

toothpaste, cleaning products, food storage containers, paint, and a vast selection of cosmetic and electronic goods.

Although silver and silver based compounds have a long history within medicine due to their antibacterial properties, the development of modern antibiotics has largely limited their use in recent years (Wong, 2010). However, the emergence of AgNPs, with enhanced antibacterial properties, coupled with a large relative surface area; (thought to encourage their reactivity and sorption to pathogens) (You *et al,* 2012) has revived the use of silver in medical, food storage, textile and environmental applications (Abou El-Nour *et al,* 2010). The antibacterial activities of several antibiotics are reported to be enhanced in the presence of AgNPs (Shahverdi *et al,* 2007), which have been reported to exhibit noticeable effects on typically resistant pathogens such as *Pseudomonas aeruginosa, E. coli O157:H7* and *Streptococcus pyogenes* (Humberto *et al,* 2009). Antiviral properties of AgNPs have also been demonstrated, with their ability to inhibit; HIV-1 replication (Elechiguerra *et al,* 2005), hepatitis B (Lu *et al,* 2008) and herpes (Baram-Pinto *et al,* 2009).

Other medical applications include therapeutics, including: wound healing (Fong and Wood, 2006), silver-impregnated catheters (Tennenberg *et al,* 1997), orthopaedics (Alt *et al,* 2004) and surgical mesh (Cohen *et al,* 2007) and diagnostic therapy, including surface enhanced Raman spectroscopy (SERS) and in cancer cell recognition using Ag-Au nanorods (Huang *et al,* 2008). The biocidal mode of action of AgNPs is widely regarded to be the release of Ag^{+}, evoking oxidative stress and protein/DNA damage which ultimately limit the resistance of bacteria (You *et al,* 2012).

Within textiles, AgNPs have been incorporated into nylon and silk to give antimicrobial properties (Dubas *et al,* 2006) and cotton (for biomedical applications) to offer bactericidal effects on pathogenic bacteria such as *Escherichia coli* (YeonáLee *et al,* 2007). Due to their nano-scale size, AgNPs are also able to display a powerful UV-visible extinction band which cannot be achieved in the bulk form (McFarland and Duyne, 2003). Consequently, their optical properties have been used extensively as biosensors (Haes and Duyne, 2002), and electroluminescent displays (Park *et al,* 2007) and could potentially become the building block for a novel form of optical data storage. AgNPs have also been widely been applied within microelectronics, notably to improve conductivity within electrical conductive adhesives (Pothukuchi *et al,* 2004), and printed

electronics due to their high conductivity and low temperature processing requirements (Li *et al,* 2005). With a catalytic activity unique to that of bulk silver, AgNPs can be used to enhance numerous reactions, including; the bleaching of organic dyes (Kohler *et al,* 2008), the oxidation of styrene (Xu *et al,* 2006) and to bore nanoholes within silicon (Tsujino and Matsumura, 2005).

1.4.5 AgNPs in the environment

Although no adverse human health effects have been reported following the use of consumer products containing AgNPs, their manufacture, use and disposal will almost certainly cause a release of AgNPs and dissolved silver into the environment (Gottschalk *et al,* 2009). For example, Benn and Westerhoff (2008) detected the leaching of almost all silver from AgNP containing, commercially available socks following four washes in ultrapure water. Similarly, Lorenz *et al* (2012) demonstrated how different AgNP containing textiles released different forms of silver at different rates; with AgCl identifieed as the most frequently observed chemical in the washwater, whilst leached nanoparticles were predominantly found in an agglomerated form.

As with other compounds of silver, estimates and quantification of AgNPs in the environment is challenging. Whiteley *et al,* (2013) estimated that in the UK, a total of 8.8 tonnes of AgNP is released (from AgNP containing products) into wastewater systems per year. Using a modelling system, Whiteley *et al* (2013) estimated a AgNP concentration of 0.10 ng/L in UK natural waters following their release from wastewater treatment plants. Although this estimate is lower than modelled figures for Europe (0.7 ng/L) (Gottschalk *et al,* 2009) and those measured in 22 USA rivers (1.2-72 ng/L) (Wen *et al,* 2002), this estimate has the potential to rise as the use and production of AgNPs continue to grow. In addition to the leaching of AgNP from consumer products, other possible routes of entry to freshwater systems include; direct, point source release upon manufacturing, the deposition of AgNPs suspended in the atmosphere and runoff from AgNP- contaminated soils and landfills (You *et al,* 2012). Although sewage sludge has the potential to bind a large proportion of AgNPs in wastewaters (Blaser *et al,* 2008), the majority of sewage sludge treatment centres are not designed for the removal of nanoparticles. In the UK, Germany and the USA, sewage sludge is widely used as a

fertiliser within agriculture. Sewage sludge containing AgNPs has shown to have detrimental effects upon soil microorganisms (Schilch *et al,* 2013) and may also contribute to freshwater environments via runoff.

Although the implications of AgNPs in the environment are still unclear, they are likely to persist and accumulate (Fabrega *et al,* 2011). Numerous capping agents (i.e. citrate, PVP and PEG) are designed to reduce the aggregation/agglomeration of particles whilst controlling their shape and ultimately their functionality. There is a large diversity of AgNPs under production and use which vary with respect to their physico-chemical properties (e.g. size, morphology, surface coating, charge), and thus it is likely that not all AgNPs will behaviour similarly in the environment. Understanding the environmental fate and behaviour of AgNP containing materials is further complicated by the coexistence of silver in nanoparticle, aggregated/agglomerated nanoparticle and soluble ion form (Benn and Westerhoff, 2008). The dissolution of AgNPs is related to their large surface area, enabling greater exposure to oxygen and allowing oxidation, ultimately resulting in the slow release of Ag^+ (Barriada *et al,* 2007). Rates of AgNP dissolution in aquatic environments are dependent on biotic factors such as dissolved oxygen content and pH (Liu *et al,* 2010) in addition to the physico-chemical properties (e.g. size, shape, morphology and coating) and concentration of AgNPs. Although the exact kinetics of AgNP dissolution are not fully understood, Liu and Hurt (2010) demonstrated that under neutral pH conditions, 5 nm AgNPs took several months to fully dissolve. In regards to risk and toxicity, the dissolution of AgNP to form Ag^+ and soluble complexes would allow comparisons with existing environmental risk and toxicity data for conventional forms of silver (Liu and Hurt, 2010).

The aggregation/agglomeration or stabilisation of AgNPs in aquatic environments is similarly dependent on biotic factors, most notably; pH, ionic strength and natural organic matter (NOM). Bradway *et al* (2010) observed greater aggregation in low pH and high ionic strength environments (particularly those containing divalent cations such as Ca^{2+}), whilst the chemistry of different capping agents showed a profound effect on AgNP aggregation potential. Delay *et al* (2011) also recorded greater destabilisation of AgNPs under high ionic strength conditions; however, they also observed this to be significantly offset in the presence of NOM, which promoted a more stable AgNP suspension. This can be explained by the NOM 'coating' generated on the surface of

AgNP through adsorption, which altered AgNP surface charge and ultimately causing steric repulsion between particles. The rate of AgNP aggregation/agglomeration will influence the particle size that organisms are potentially exposed to and could in turn, affect toxicity. Understanding the processes of dissolution and aggregation/agglomeration are crucial areas of research as they will determine whether AgNP remain in particle form in the environment. AgNPs in particle form are likely to present unique environmental risks due to their novel size and physicochemical properties.

1.4.6 Toxicity of AgNPs

Of the AgNP ecotoxicity studies conducted, a high number is in relation to fish species and in particular, zebrafish *(Danio rerio)*. Lee *et al* (2007) observed the uptake of AgNPs (5-46nm) by zebrafish embryos in the early development stage, whilst George *et al* (2012) demonstrated toxicity towards embryo gill cell lines when exposed to AgNP of various shapes (nanospheres, plates and wires) and sizes (7-60nm). Asharani *et al* (2008) recorded a concentration (0-100µg/L) dependent change in zebrafish heart rate, hatching rate and mortality upon AgNP (5-20 nm) exposure for 72 hours. Further responses observed included abnormal body axes, and slow blood flow, whilst AgNP distribution was detected in the brain, heart, yolk and blood of embryos (Asharani *et al,* 2008). Bar-Ilan *et al* (2009) recorded a size dependent toxicity of AgNPs in relation to zebrafish, whereby particles of sizes between 3-100 nm displayed progressively higher LC_{50} values (ranging from 93.1µM to 137.2 µM) with an increase in size. AgNP are also readily taken up in the gills of rainbow trout (Farkas *et al,* 2010) and perch (Bilberg *et al,* 2010) which can lead to internal hypoxia and cytotoxic effects.

Within invertebrate testing, numerous studies have assessed the toxicity of AgNP towards *D. magna* to investigate aquatic toxicity. Acute toxicity testing has demonstrated the ability of AgNPs to cause toxicity towards *D. magna*, however the level of toxicity has shown to vary between particles. Hoheisel *et al* (2012) reported increasing LC_{50} values (4.31-30.36 µg/L) with a decrease in AgNP size (10-50nm), however, alternate studies (Li *et al,* 2010) found no difference in the toxicity of differently sized AgNPs (36, 52 and 66nm), all recording LC_{50} values of 3-4 µg/L. In relation to surface coatings, Poynton *et al* (2012) found PVP (poly vinyl pyrrolidone) coated AgNPs to stimulate metal responsive and DNA damage genes in *D. magna*

(following 24h exposure) that were not evident in citrate-coated AgNP exposures. Furthermore, Zhao and Wang (2011) recorded significant differences in *D. magna* mortality between differently coated AgNPs (lactate, PVP and sodium dodecylbenzene – with 48h LC_{50} values of 28.7, 2 and 1.1 µg/L respectively). Soluble Ag was found to be the primary cause of observed toxicity (LC_{50} values comparable to that of $AgNO_3$). Therefore, differences in toxicity were attributed to the dissolution of AgNP into soluble Ag, which was ultimately influenced by surface coating.

Despite finding no *D. magna* mortality following acute AgNP exposure (48h) at a high concentration (500 µg/L), Zhao and Wang (2011) observed significant inhibition of growth and inhibition during chronic (21 day) exposures. Additionally, Gaiser *et al* (2011) recorded no inhibition of growth following chronic (21 days) AgNP exposure (1 µg/L), although they did record reduced moulting. Ingestion is indicated to be the primary mode through which *D. magna* take up AgNPs, with Zhao and Wang (2012) discovering over 60% of particles distributed in the gut and also found efflux rates to be low, suggesting that the elimination of AgNP by *D. magna* was difficult (Zhao and Wang, 2010). Changes in *D. magna* gene expression recorded by Poynton *et al* (2012) (described above) were attributed to the damaging effects of AgNP upon protein metabolism. Park *et al* (2010) discovered significant DNA strand breaks in *D. magna* exposed to 50 nm AgNP for 24 hours, providing further evidence towards their potential genotoxicity.

The soil nematode, *Caenorhabditis elegans* represents another popular invertebrate model within ecotoxicology. As with other test species, AgNP coatings were also found to have a profound effect on their toxicity towards *C. elegans.* Meyer *et al* (2010) observed growth inhibition and internalisation of both PVP and citrate coated AgNPs, but hypothesised that only the latter exert nano-specific toxicity (as PVP toxicity was attributed to dissolution). Yang *et al* (2011) also recorded growth inhibition between differently coated AgNPs (PVP, citrate, gum arabic) and similarly reported a direct correlation between AgNP toxicity and silver dissolution. Although smaller sized particles are generally thought to exert a greater toxicity, neither Yang *et al* (2011) (with particles ranging 8-75nm) nor Ellegaard-Jensen *et al* (2012) (using 1nm and 28nm) found this to be the case for AgNP exposed *C. elegans.* Genomic analysis of *C. elegans* following AgNP exposure (20nm for 24h, concentrations 0.05-0.5 mg/L) revealed an

increased expression of superoxide dismutase-3 *(sod-3)* and abnormal dauer formation protein *(daf-12)* leading Roh *et al* (2009) to speculate that reproductive failure was linked to oxidative stress. Lim *et al* (2012) also recorded an up-regulation of glutathione S-transferase (GST) enzyme and a decline in reproductive potential.

A handful of publications have also demonstrated the effects of AgNPs on freshwater algae. Oukarroum *et al* (2012) detected a strong decrease in chlorophyll content, viable algal cells, increased ROS formation and lipid peroxidation in the green algae, *Chlorella vulgaris* and *Dunaliella tertiolecta.* Although Navarro *et al* (2008) found AgNP toxicity (inhibitory effects on photosynthesis) to be mediated by Ag^{+}, it did not account for all toxicity, indicating a nano-specific source of toxicity.

Current publications demonstrate the complexities involved in predicting the ecotoxicological effects of AgNPs. Existing studies have demonstrated that capping agents and nanoparticle size have an influence on AgNP toxicity; however, uniform patterns of toxicity between species are not always evident. It is also unclear whether AgNPs exhibit modes of action distinct to those found in Ag ions. A number of studies cite the dissolution of silver to be the primary source of AgNP toxicity, whilst others have uncovered novel modes of AgNP action, most notably the production of reactive oxygen species (ROS), (Völker *et al,* 2013). Although the NP-mediated ROS production is not fully understood, the presence of pro-oxidant groups on their surface and interaction with cells are often cited as likely causes. It is clear that generalisations cannot be made in regards to the ecotoxicological effects of AgNP, highlighting the importance for continued research.

1.5 Titanium dioxide

1.5.1 Titanium dioxide nanoparticles

Titanium is the ninth most abundant element in the Earth's crust, with an average concentration of 4400 mg/kg (Shi *et al,* 2013). Titanium is readily oxidised and thus does not exist in its metallic form in nature. The naturally occurring oxide of titanium, titanium dioxide (TiO_2) is a white, solid, inorganic, poorly soluble particulate that commonly exist in the crystalline mineral forms, anatase and rutile. TiO_2 has long been

used as a pigment to add whiteness or opacity to a number of consumer products such as toothpaste and paint, whilst its photocatalytic properties also enable the breakdown of an array of organic compounds when exposed to sunlight (Rezaei and Mosaddeghi, 2006). As a relatively chemically inert material, TiO_2 is generally considered to be a non-hazardous material by the United Nations Globally Harmonised System of Classification and Labelling of Chemicals (GHS). However, the International Agency for Research on Cancer (IARC) concluded that TiO_2 was possibly carcinogenic to humans (IARC, 2010).

The photocatalytic capabilities of TiO_2 are significantly heightened at the nano-scale, due to an increase in redox potential, related to size and crystal structure. Their predominantly anatase (rather than rutile) composition are also cited to increase their catalytic activity (Sayes *et al,* 2006). The photocatalytic properties of TiO_2NP allow them to be used as; semiconductors (Kotov *et al,* 1995), in the treatment of contaminated water (Mahmoodi *et al,* 2007) and within solar cells (Benkstein *et al,* 2003) as nanocrystaline structures. TiO_2NPs are also extensively used for self-cleaning purposes within; textiles for the removal of stains (Montazer and Pakdel, 2011) and glass, such as windows and car windshields (Kafizas *et al,* 2009) whilst their anti-fogging capabilities are used in car mirrors (Shi *et al,* 2013). Consequently, TiO_2NPs have emerged as a popular nanomaterial and are produced on a mass scale, globally. Although not as fully implemented as AgNP, the use of TiO_2NPs for medical purposes is now being explored. Both photodynamic therapy (PDT) and sonodynamic therapy (used in cancer treatment) have revealed greater treatment efficacy in the presence of TiO_2NPs (Miyoshi *et al,* 2011). Although TiO_2NP have been shown to exhibit antibacterial properties (in relation to *E. coli*), they are considered to be exclusively phototoxic (Brunet *et al,* 2009). The Woodrow Wilson inventory on nanotechnology-based consumer products currently (as of August 2016,) lists 92 products containing titanium dioxide nanomaterials, including; paints, sunscreens, shampoo, sports equipment, clothing and numerous electronic goods to name a few.

1.5.2 TiO_2NPs in the environment

Both natural and anthropogenic sources of TiO_2 exist in the environment. Natural sources are predominantly due to the weathering of plutonic and metamorphic rocks

and present as the minerals rutile and ilmentie (Sharma, 2009). Given the global scale at which TiO_2NP are produced, anthropogenic sources are likely to be released into the environment. Exposure modelling conducted by Mueller and Nowack (2008) suggests that the runoff, application and disposal of paints and cosmetics will account for the two largest anthropogenic sources of TiO_2NPs in the environment (25% and 60% of total TiO_2NP released, respectively).

During the weathering and subsequent oxidation of the surface layers of paint, TiO_2NPs are likely to become available in the environment. Kägi *et al,* (2008) could detect TiO_2NPs in the façade runoff and nearby surface runoff of a relatively recently painted building. TiO_2NPs ranging from a few ten to a few hundred nanometres in size were detected at relatively high environmental concentrations (around 16µg/L). Conversely, following the exposure of panels coated in TiO_2NP paint to simulated weathering conditions, Al-Kattan *et al* (2014) found a low release of TiO_2NP (less than 1.5 µg/L) into the nearby aquatic environment. However, under UV exposure they observed 100-times greater release of Ti from TiO_2NP paint (in the form of individual NPs, large aggregates and large paint fragments) compared to pigment TiO_2 paint, indicating a photocatalytic degradation of the organic paint matrix.

Sunscreens are at the forefront of TiO_2NP cosmetic products and despite rigorous testing in regards to human safety, little attention has been paid to the fraction of sunscreens potentially lost to the environment during their life cycle (Botta *et al,* 2011). Water immersion or sand abrasion of sunscreen-applied skin could lead to changes in sunscreen properties or their attenuation (Stokes and Diffey, 1999; Stokes and Diffey, 2000), which could ultimately lead to environmental releases. Data regarding the form in which TiO_2NPs may exist in sunscreen residues are limited. Labille *et al* (2010) found an aged TiO_2 based nanocomposite used in sunscreen to form a stable suspension of colloids from 50-700 nm in water.

1.5.3 Toxicity of TiO_2NPs

As with AgNPs, both fish and *D. magna* prove popular model organisms within TiO_2 NP ecotoxicity testing. Federici *et al* (2007) observed no mortality in rainbow trout (*Oncorhynchus mykiss*) when exposed to up to 1 mg/L TiO_2NP, however they did find

effects in the gills, brain, intestine and liver including; thickening of the lamelle, decrease in Na^+ K^+-ATPase activity, increase in glutathione levels and thiobarbituric acid reactive substances and minor changes to fatty acids. Following dietary exposure (10 and 100 mg/kg), Ramsden *et al* (2009) also found increases in thiobarbituric acid reactive substances in the gills and intestine and a 50% inhibition of Na^+ K^+-ATPase activity in the brain. Although no influence on growth or nutritional performances were detected, TiO_2NP accumulation in the gills, gut, brain, liver and spleen were observed, which proved to be persistent even after exposure had terminated. Following intravenous injection of TiO_2NP to rainbow trout, Scown *et al* (2009) witnessed significant accumulation in the kidneys, however no loss in function. Studies using carp (*Cyprinus carpio*) have demonstrated how TiO_2NPs could potentially enhance the bioaccumulation of other substances such as cadmium (Zhang *et al,* 2007) and arsenate (Sun *et al,* 2007).

Although no effects on hatchability, survival or formation of zebrafish embryos have been observed up to a concentration of 10 mg/L TiO_2NP (Chen *et al,* 2011), Bar-Ilan, (2012) demonstrated their photo-dependent toxicity. More specifically, TiO_2NP exposure led to zebrafish embryo malformation and in some cases, death, however, only upon the illumination of the exposure vessels during the test period. Similarly, Reeves *et al* (2007) found TiO_2NP (0.1-1000 µg/L) alone to have little effect on the cell viability of goldfish skin cells *in vitro*, however, a dose dependent decrease was recorded in the presence of increasing UVA dose. Furthermore, greater DNA damage was also reported in UVA treatments, indicating that TiO_2NP toxic effects were likely to be photo-dependent and due to the formation of hydroxyl radical.

TiO_2NP exposures have revealed both acute and chronic toxicity towards *D. magna.* Bang *et al* (2011) found toxicity to be a factor of both TiO_2NP crystal form and size, indicating anatase particles to be significantly more toxic than rutile and reporting the smallest particle (anatase, 21nm) as the most toxic. During chronic testing (21 days), *D. magna* died before replicating when exposed to a concentration of 10 mM TiO_2NP. Reproduction was also found to be affected upon chronic (21 day, concentration 0.1-5 mg/L) TiO_2NP exposure by Zhu *et al* (2010), along with growth and incidents of mortality. They also concluded that once accumulated in *D. magna,* TiO_2NP are not easily eliminated, potentially affecting food uptake and ultimately hampering growth and reproduction.

In contrast, TiO_2NP have also demonstrated low toxicity towards *D. magna* i.e. surviving exposures up to 20 mg/L TiO_2NP have been reported (Heinlaan *et al,* 2008) and no significant changes in behavioural responses have been observed (Lovern and Klaper, 2006) nor any genotoxic effects (Lee *et al,* 2009) following exposure. Incidents of low toxicity could be attributed to the photo-dependent nature of TiO_2NPs as demonstrated by Hund-Rinke and Simon, (2006) who observed increased toxicity in *D. magna* exposed to pre-illuminated particles. Similarly, Ma *et al* (2012), found TiO_2NP *D. magna* toxicity to increase by two to four orders of magnitude under simulated solar radiation as opposed to ambient laboratory light.

TiO_2NP toxicity to freshwater algae has been assessed using the species *Pseudokirchneriella subcapitata, Desmodesmus subspicatus and Chlamydomonas reinhardtii. P. subcapitata* is generally considered to be the most sensitive algal species (Menard *et al,* 2011) and incidents of growth inhibition upon TiO_2NP exposure have been widely observed (Hartmann *et al,* 2010; Aruoja *et al,* 2009; Warheit *et al,* 2007). Although no clear relationship between particle size and toxicity is evident for *P. subcapitata* studies, higher specific surface area is thought to increase TiO_2NP toxicity (Menard *et al,* 2011). In addition to growth inhibition, Wang *et al* (2008) also revealed lipid peroxidation and the transient up-regulation of the stress response genes *sod1, gpx, cat*, and *ptox2* in the unicellular green algae *C. reinhardtii.*

1.6 *Lumbriculus variegatus*

1.6.1 Biology and ecological role

Lumbriculus variegatus, commonly referred to as the California blackworm, is a freshwater oligochaete which typically inhabits shallow habitats such as; ponds, lakes, slow flowing rivers and marshes across North America and Europe. Although *L. variegatus* reach a length of 5-10 cm (or 100-250 body segments) in the wild, those cultured under laboratory conditions are generally smaller, at 4-6 cm (100-150 body segments). Anterior segments of adult *L. variegatus* are generally wider and more manoeuvrable than posterior segments, whilst displaying darker pigmentation. As a hermaphrodite species, both male and female sex organs of sexually mature (and typically wild) worms are located within the first specialised 1-8 anterior segments which

also accommodate; a conical prostomium (mouth) and a muscular pharynx. Although posterior *L. variegatus* segments appear moderately uniform, subtle differences in structure and function influence the majority of organ systems.

In the wild, *L. variegatus* typically inhabit muddy sediments, into which they burrow their head segment, leaving the tail extended in the water column to facilitate specialised gas exchange. The absence of longitudinal and circular muscles within the dorsal wall of *L. variegatus* tail segments permits gas exchange across the epidermal layer, directly to the dorsal blood vessel (Drewes, 2004). By bending the tip of the tail at a right angle to the rest of their body, *L. variegatus* can position their pulsating dorsal blood vessel within close proximity to air, thus optimising gas exchange. Blood is transported within a closed system of vessels and capillaries, whereby rhythmic contractions of the dorsal vessel transport blood, segment by segment from the posterior end to the head (Drewes, 2004). Although prone to predation (due to sediment burrowing, leaving their tail extended in the water column), the innate and rapid escape reflexes of *L. variegatus* allows survival (Drewes and Fourtner, 1989; Zoran and Drewes, 1987). Drewes and Fourtner (1989) observed *L. variegatus* to rapidly withdraw its tail following an abrupt decrease in light intensity or upon encountering moving shadows. Touch-stimuli or substrate vibration also provokes escape reflexes in anterior segments, leading to rapid head shortening or withdrawal (Zoran and Drewes, 1987). Whilst in open waters, touch-stimuli also incite distinctive anterior and posterior behaviours in the form of body reversal and helical swimming, respectively (Drewes, 1999).

L. variegatus, together with other benthic species, embody an important link within the freshwater food web (Liebig *et al,* 2004). As non-selective feeders, *L. variegatus* primarily consume decaying vegetation, microorganisms and subsurface sediments, whilst they act as prey for bottom-feeding fish and sediment-probing birds (Wallace and Webster, 1996). The ingestion of sediments from varying depths, followed by the disposal of gut contents on the sediment surface accelerates the rate of sediment and pore water burial (Robbins, 1986). Sediment re-working and bioturbation during feeding and foraging has a profound effect on the ecological properties of sediments and overlying waters (McCall and Tevezs, 1982).

1.6.2 Reproduction and autotomy

Although the details of sexual reproduction are not entirely clear for wild *L. variegatus*, it is thought to resemble that of earthworms whereby worms exchange sperm during copulation. Transparent cocoons each containing 4-11 fertilised eggs are subsequently formed, which after roughly two weeks of direct embryonic development, produce small worms of approximately 1cm in length. Laboratory cultured *L. variegatus* multiply exclusively via asexual reproduction, achieved through fragmentation and subsequent regeneration, also known as architomy (Martinez *et al,* 2006). Adult worms spontaneously divide into two or more body fragments which are then able to develop either a new head or tail via epimorphic regeneration to eventually form a clone worm of standard size and physiology. Similarly, in response to direct damage or upon encountering damaging stimuli, *L. variegatus* are also able to rapidly self-amputate body parts in a process called autotomy (Lesiuk and Drewes, 1999). Following autotomy, remaining body fragments survive and proceed to rapidly generate missing head or tail segments (Stephenson, 1939).

1.6.3 Use in ecotoxicological testing

L. variegatus are widely regarded as an ideal freshwater macroinvertebrate for the ecotoxicological testing of potentially harmful substances, including; metals (Ankley *et al,* 1994), organic chemicals, such as polychlorinated biphenyls (PCBs) (Fisher *et al,* 1999) and polycyclic aromatic hydrogen (PAHs) (Leppanen and Kukkonen, 1998), dioxins (West *et al,* 1997), organobromine compounds (Ciparis and Hale, 2005) and herbicides (Maenpaa *et al,* 2003). Numerous attributes make *L. variegatus* an ideal model organism. Namely, their intimate association with freshwater sediments. As a sedentary species, highly reliant on sediments for a number of life functions (Davis and Lathrop, 1992), the likelihood of *L. variegatus* interacting with sediment-bound contaminants, via contact, ingestion and exposure to pore water and overlying water is high. Due to its role in bioturbation and the food web, *L. variegatsus* are thought to strongly influence the bioavailability of contaminants. As an ecologically relevant species, their decline could ultimately cause shifts within ecological structure and function.

From a laboratory perspective, the affordability and straightforward maintenance of *L. variegatus*, together with their ability to rapidly multiply, make them highly favourable. Additionally, they are suitable for both short and long term exposure scenarios whilst providing sufficient tissue mass to assess bioaccumulation and biochemical end points. The well-defined behavioural responses of *L. variegatus* also serve as valuable end points within ecotoxicological testing.

The Organisation for Economic Co-operation and Development (OECD) test guideline 225 is currently in place for the testing of chemicals using spiked sediments with *L variegatus* as a model organism. Through 28 day, static exposures, OECD 225 is designed to determine the effects of prolonged exposure on *L. variegatus* reproduction and biomass and has been used to assess the toxicity of the parasiticide, ivermectin (Egeler *et al,* 2010); PACs (Paumen *et al,* 2008) and sediments with low-level anthropogenic contamination (Höss *et al,* 2010).

Given the extent to which *L. variegatus* have been used in the screening of 'conventional' contaminants, it is unsurprising that they have become more popular in the ecotoxicological testing of nanomaterials, including carbon nanotubes (Petersen *et al,* 2008), fullerenes (Pakarinen *et al,* 2011), silver (Coleman *et al,* 2013) and titanium dioxide (Hartmann *et al,* 2012). The tendency of nanomaterials to aggregate and subsequently undergo sedimentation in the environment makes *L. variegatus* a species of increasing interest given their close association with both sediment and water.

1.7 The MARINA project

This research was conducted as part of the MARINA (Managing Risks of Nanomaterials) project. Funded by the Seventh Framework Programme, MARINA incorporates over 40 institutions across Europe with the primary aim of developing and validating the risk management methods for engineered NPs. The four central themes of MARINA include; *Materials, Exposure, Hazard and Risk.* This project was part of work package 10, concerned with NP hazard assessment in aquatic, sediment and terrestrial environments. Within this project, the main MARINA-led objectives related to sediment toxicity testing and the suitability of current sediment toxicity protocols (OECD 225) for use with NPs. NP characterisation, aquatic toxicity, abiotic factors, behavioural and

mode of toxicity studies were also used to gain a greater understanding into the toxicity of NPs towards *L. variegatus.*

1.8 Aims

The overarching aim of this research was to identify the potential hazards posed by two reference engineered NPs: silver (NM-300K) and titanium dioxide (NM-104) towards the freshwater oligochaete, *L. variegatus.* The suitability of standard sediment toxicity protocols were also investigated in relation to the above mentioned NPs. To achieve this, the following were addressed:

- Generate characterisation data for the physicochemical properties of NM-300K (Ag) and NM-104 (TiO_2) in environmental test medium and compare to those generated by the manufacturers;
- Assess the acute toxicity of NPs to *L. variegatus* exposures in aquatic environmental test medium via assessment of lethality and identify LC50 values;
- Investigate the effects of acute NM-300K and NM-104 exposure on the intrinsic survival behaviours of *L. variegatus;*
- Investigate the influence of abiotic factors (pH, ionic strength and organic matter content) on toxicity of NM-300K toxicity towards *L. variegatus.*;
- Determine whether NM-300K and NM-104 stimulate oxidative stress in *L. variegatus* measured via assessment of the level / activity of the antioxidant enzymes (superoxide dismutase and catalase) and lipid peroxidation (via the TBARS assay);
- Assess the suitability of current 28-day sediment exposure protocols for *L. variegatus* (OECD 225) to NM-300K and NM-104;
- Observe the effects of chronic (28 day) NM-300K and NM-104 sediment exposure towards *L. variegatus* survival, reproduction and biomass;
- Investigate the uptake of NM-300K by *L. variegatus* following sediment exposure using field flow fractionation coupled with inductively coupled mass spectroscopy (FFF-ICP-MS).

Chapter 2: Characterisation of engineered Ag (NM-300K) and TiO_2 (NM-104) nanoparticles.

2.1 Introduction

The physico-chemical properties of NPs determine their toxicity. In parallel to hazard studies, it is essential to characterise NP physico-chemical properties in order to identify which properties may confer toxicity. In instances where NPs share the same composition (e.g. silver or titanium dioxide), other physico-chemical properties can be vastly different, such as crystalline structure, particle size distribution, morphology, agglomeration/aggregation status, surface coating, charge, surface area and surface reactivity – all of which intrinsically influence toxic potential (Warheit, 2008). Despite this, the risks of NPs are typically assessed on their chemical composition, however, efforts to increase the emphasis of thorough physico-chemical NP characterisation within risk assessments have been made (Stone *et al,* 2014).

Efficient NP characterisation not only provides information in relation to risk but also facilitates the development of safer NPs for the future. For example, using a 'safe-by-design' approach, Xia *et al* (2011) found that the sub-lethal and lethal effects of ZnO NPs towards rodent lungs and zebrafish embryos could be alleviated when doped with iron. Through the collection of characterisation data, it is possible to predict the toxicity of NPs through the use of quantitative structure-activity relationship (QSAR) *in silico* models (Burello and Worth, 2011). The implementation of efficient NP characterisation is consequently an area of continued interest and development. Through the use of various characterisation techniques in environmental media and biological systems, it is possible to link NP properties to effects within organisms in a reliable and reproducible manner.

Insufficient NP characterisation has been highlighted as a major issue within nanotoxicology studies over recent years (Murdock *et al,* 2007, Warheit *et al,* 2008; Cong *et al,* 2011). Although extensive characterisation of all NP properties is unrealistic in regards to both cost and time, it is generally agreed that some key properties, such as, size, shape, agglomeration/aggregation, charge, composition, surface area and surface chemistry should be routinely considered (Powers *et al,* 2006, Moudgil, 2004, Cong *et al,* 2011, Murdock *et al,* 2007).

Understanding the fate and behaviour of NPs in the environment is a challenging task given the high number of variables to consider (e.g. natural organic matter, pH, ionic strength and temperature), however, most NPs will undoubtedly undergo changes in relation to particle size distribution, aggregation/agglomeration state and surface chemistry (Cong *et al,* 2011; Ju-Nam and Lead, 2008) when introduced in the environment. While a number of studies acknowledge the importance of characterising the previously mentioned NP properties of interest, they tend to refer to their original status, as manufactured, or as suspended in simple media such as Milli-Q water. Although this may provide useful data such as confirming manufacturers' measurements, it does not provide characterisation information for NPs within ecotoxicological testing media.

Similar to true environmental scenarios, NPs are likely to undergo significant changes in laboratory settings when transferred between dispersion media such as Milli-Q water and those used within testing such as artificial freshwater, seawater, sediment and soil *etc.* (Cong *et al,* 2011). Such changes have the potential to influence NP bioavailability and, therefore toxicity towards organisms and are consequently extremely important to interperate the results obtained from ecotoxicological testing of NPs. Given the uncertainty surrounding the behaviour of NPs in the environment, there are calls for research to focus on assessment of their fate (mobility, aggregation, complexation) and behaviour (in relation to gradients of pH and ionic strength) in order to enhance the relevance of future ecotoxicological studies (Tiede *et al,* 2009).

A plethora of techniques exist for the characterisation of NP properties, each with its own merits and limitations. One method cannot be indentified to be 'the best' for a certain NP; rather, they are used in combination to achieve a complimentary, representative analysis (as each technique characterise different properties). With this in consideration, a number of characterisation techniques were used within this investigation, each of which is discussed in the following sections.

2.1.1 Dynamic Light Scattering

Dynamic light scattering (DLS) has been identified as a useful technique for the analysis of size and size distribution of nanomaterials in suspension (Powers *et al,*

2006) and has been applied widely within biology, physics and chemistry since 1975 (Berne and Pecora, 2000). DLS is based on the theory that particles within a suspension will undergo Brownian motion (whereby fluid molecules collide with particles, causing them to move) (Berne and Pecora, 2000). During DLS, a light beam is passed through the sample; the particles subsequently cause the light direction and intensity to become altered due to a scattering process (Hulst and Van De Hulst, 1957). The intensity of the scattered light fluctuates according to the size of particles within the sample. The diffusion coefficient is calculated from these intensity fluctuations, from which the hydrodynamic diameter can be determined (for spherical particles) using the Stokes-Einstein equation (Lim *et al,* 2013).

The speed and ease at which samples can be processed is one of the main advantages of DLS (Lim *et al,* 2013). Furthermore, it can be used to measure narrow particle size distributions within the range of 2-500 nm (Tomaszewska *et al,* 2013). Although widely used within nanoparticle characterisation, DLS also has its drawbacks, namely when processing samples with large size distributions or multimodal distribution. Within polydispersed samples, it is possible that individual particles or smaller aggregates/agglomerates will be masked by larger aggregates/agglomerates and remain undetected, leading to larger average particle size readings, which do not reflect the true size distribution of the NPs in suspension (Tomaszewska *et al,* 2013). Interferances, most commonly caused by dust particles, can influence scattering intensity (in comparison to smaller particles) during DLS which can also adversely affect sizing measurements (Brar and Verma, 2010). Furthermore, DLS assumes particles to be spherical which can lead to an underestimation of particle size in some instances (Barr and Verma, 2010). DLS produces a polydispersity index (PdI) which relates to the width of overall particle distribution (with values close to 0 indicating a highly monodispere sample and above 0.7 indicating a very broad distribution). Despite its limitations and the fact that it does not provide full characterisation of the nanoparticle dispersion, DLS is a very valuable tool for the monitoring of agglomeration/aggregation behaviour (Hassellöv *et al,* 2008) and has shown longevity as a characterisation technique since its first use with particle suspensions in the 1970's (Xu, 2008).

2.1.2 Differential centrifugal sedimentation

Although particle characterisation is possible via gravitational sedimentation, smaller particles (including NPs) rarely settle by this means unless very dense (Laidlaw and Steinmetz, 2005). By applying centrifugal forces, it is possible to extend sedimentation analysis to much smaller particles. Differential centrifugal sedimentation (DCS), also referred to as two-layer sedimentation (Laidlaw and Steinmetz, 2005), is an established NP characterisation technique which produces extremely high resolution size distributions, especially within the size range of 3nm to 80 µm (Vegad, 2007). When particles have a different density to the fluid in which they are suspended, their sedimentation rate will depend upon the following (Vegad, 2007):

- Gravitational field strength
- Difference in density
- Fluid viscosity
- Particle size and shape

During differential centrifugation, particles (in suspension) are introduced to the top of a clear liquid in a column and settle according to Stokes' Law. A detector beam (either a light or x-ray beam), which passes through a known distance from the liquid surface, initially reads at maximum intensity (i.e. there are no particles blocking the beam). As particles begin to settle, they reach the detector beam leading to a loss in intensity- this loss in intensity is related to particle concentration. During differential centrifugation analysis, only particles of one specific size are being recorded by the detector beam at any given time, hence why the method is referred to as 'differential' (Laidlaw and Steinmetz, 2005). The time at which particles pass through the detector is related to size, with larger particles passing first. Once all particles have passed through the detector beam, the intensity returns to maximum and a plot of particle concentration against the calculated particle diameter is used to determine differential distribution (Laidlaw and Steinmetz, 2005).

In cases where dispersed particles are denser than the fluid in which they are suspended, they tend to settle rapidly in aggregated/agglomerated form rather than individually as according to Stokes' Law. This phenomenon is known as 'streaming'

and results in broad, overlapping peaks for particle size distributions. By establishing a density gradient within the fluid column, it is possible to avoid streaming and produce separated peaks for each narrow band of particle sizes. Differing sucrose concentrations are commonly employed as water based gradients and can include salts, buffers and pH to maintain compatibility with the sample (Vegad, 2007).

The obvious advantages of DCS include; the particle size range it can analyse, an ultra-high resolution (~2% peak resolution), accuracy, repeatability and high sensitivity (Vegad, 2007). Conversely, low density particles may take a long time to settle, whilst the optical properties of particles must be known to determine volume or number based size distributions. This has the potential to cause problems in instances where two or more NPs of different densities are to be examined in parallel (Fissan *et al,* 2014).

2.1.3 Zeta potential

Within an ionic solution, NPs with a net charge have an electrical double layer composed of the Stern layer (a layer of strongly bound ions to the NP surface); and an outer, diffuse layer, comprised of loosely associated ions (Clogston and Patri, 2011). As NPs move through the suspension (because of Brownian motion, gravity or an applied force), ions will either move with the NPs or, beyond a certain boundary from the particle, remain associated with the bulk dispersant (Clogston and Patri, 2011). This 'boundary' within the diffuse layer is commonly referred to as the surface of hydrodynamic shear or the slipping plane. The electric potential here is known as the zeta potential (Revil *et al,* 1999). The measurement of zeta potential is based on the principal of electrophoresis, whereby an electric field is applied across the dispersion, causing charged particles to navigate towards the electrode of opposite polarity (Jiang *et al,* 2008). By passing a laser beam through the sample undergoing electrophoresis, a calculation of electrophoretic mobility can be determined using laser dopler velocimetry (LDV), from which the zeta potential is generated (Hassellöv *et al,* 2008).

As there is no satisfactory means by which to directly determine the surface charge of particles, measurements of zeta potential (which are closely related to NP surface charge) are commonly utilised as an indicator of charge, and to determine colloidal stability (Xu, 2008). In cases where all particles have either a large positive or negative charge, they will repel one another resulting in a stable suspension. Conversely, when the zeta potential of particles is low, the tendency to form aggregates is greater. Zeta potential values typically range from +100 mV to -100 mV, those between -10 and +10 mV are considered fairly neutral, whilst those greater than +30 mV or less than -30 mV are regarded as strongly cationic or anionic, respectively (Clogston and Patri, 2011).

2.1.4 Transmission electron microscopy

Transmission electron microscopy (TEM) is a widely-applied tool that enables the direct imaging of NPs in order to visualise their morphology and quantify their size, and size distribution. TEM uses a tungsten filament to produce an electron beam in a vacuum chamber. Electrons are passed through an electromagnetic field to focus the beam, which is subsequently passed through an ultra-thin sample to produce an image. In cases where samples are less dense, more electrons may pass through, producing a brighter image. Denser samples allow fewer electrons to pass through, resulting in a darker image. TEM provides high resolution images down to 0.2 nm (Reimer, 1991) and is commonly the first tool used in the determination of NP size and size distribution due to its ability to quickly and easily count a large number of particles (Woehrle *et al,* 2006). Limitations related to TEM analysis are commonly related to polydisperse samples, whereby NP agglomeration/aggregation or sample fractionation may occur during drying (Tomaszewska *et al,* 2013) leading to a misinterpretation of size and size distribution. Other disadvantages include the need for precise and often complicated sample preparation and cost, which is often high.

2.1.5 Energy dispersive x-ray spectroscopy

Energy dispersive x-ray spectroscopy (EDS) is an analytical capability which is often coupled with microscopy techniques for the characterisation of NPs. After electrons, the signal most commonly measured in the electron microscope is that of characteristic x-rays (Zaluzec, 2009). When exposed to an electron beam during TEM analysis, samples emit characteristic x-rays due to the excitation of inner shell electrons (Shindo and Oikawa, 2002). By placing x-ray detectors such as EDS within TEMs, it is possible to determine the elemental composition of individual points within samples or create x-ray maps of materials. Confirming the elemental composition of NPs is an important parameter to consider within nontoxicity studies – e.g. to confirm the presence of particles and assess any potential contamination. EDS is commonly used due to its; ability to detect elements across the periodic table, quick analysis and spatial resolution of <10 nm (Hassellöv *et al,* 2008).

2.1.6 Dissolution

When introduced to the aqueous phase, particles may undergo dissolution to form a homogenous mixture (Borm *et al,* 2006). Dissolution is a dynamic process, dependent upon particle solubility, which in turn is influenced by composition, surface properties, size and the media conditions (pH, salinity, temperature etc.) in which particles are suspended. The dissolution of NPs is an important parameter to consider when characterising NPs as it can affect their toxicity and persistence in the environment. Toxicity may arise due to particle mediated effects, via their dissolved fraction (particularly in cases where NPs are composed of elements known to have toxic effects; e.g. Ag^{+}, Zn^{2+}, Cu^{2+}), or as a combination of the two (Misra *et al,* 2012).

Although it is possible to measure dissolution directly, via ion selective electrodes (ISE) and single particle inductively coupled plasma-mass spectroscopy (SP ICP-MS), indirect techniques require the dissolved fraction to be separated from the particle suspension prior to analysis (Misra *et al,* 2012). Separation is commonly achieved using ultracentrifugation (Levard *et al,* 2011); filtration (Schmidt and Vogelsberger, 2006); dialysis (Kittler *et al,* 2010) or a combination of techniques (i.e. centrifugal ultrafiltration). Following separation, the dissolved fraction undergoes elemental

analysis via either atomic adsorption spectroscopy (AAS), ICP atomic emission spectroscopy (ICP-AES) or ICP-MS. From these techniques, ICP-MS offers the greatest sensitivity (less than 1 part per trillion) and a respectable sample throughput time (2-3 minutes per sample) and is thus commonly employed within NP dissolution studies.

During ICP-MS, samples (as aerosol droplets) are introduced to an argon laser which subsequently dries and dissociates the molecules. The laser also removes an electron to form singly-charged ions which are filtered through a mass spectrometer. After exiting the mass spectrometer, ions meet a detector, causing a cascade of electrons which are then amplified to create a measurable pulse. By comparing the intensity of pulses to those of the standards, it is possible to determine the concentration of the desired element.

2.1.7 Aims

Using multiple characterisation techniques, this investigation aims to establish the size, morphology, and some key physio-chemical properties (e.g. surface charge and composition) of NM-300K (Ag) and NM-104 (TiO_2) NPs. Studies were conducted in both Milli-Q water and OECD medium (OECD 225, 207) to assess whether any NP characteristics were altered when suspended in a testing medium of greater environmental relevance. NP characterisation was also performed in OECD medium containing Suwannee River Humic Acid (SRHA), as a NOM source, and in OECD medium with altered ionic strengths (50 and 950 µS/cm) to assess whether abiotic properties had any influence on NP properties and/or behaviour. Ultimately, data generated within this chapter will be used in an attempt to interpret any potential toxic effects of NM-300K and/or NM-104 toward *L. variegatus.*

2.2 Methods

2.2.1 Nanoparticles

NPs were supplied by the EU Joint Research Centre (JRC) nanomaterial repository. AgNPs, NM-300K (a sub-sample of NM-300) originate from a single batch commercially produced for the measurement and testing of hazard identification, risk and exposure assessment studies. NM-300K is a yellow/brown dispersion with a silver content of 10.16 w/w% and is reported to have 99% particles <20nm (Klein *et al,* 2011). NM-300K is highly viscous and contains the stabilising agents polyoxyethylene glycerol trioleate and polyoxyethylene sorbitan mono-laurat (Tween 20) (4% w/w% each). TiO_2NPs (NM-104) – rutile, thermal, hydrophilic, were provided in (white) powder form and quoted to have a primary particle size of 21nm and consist of an aluminium coating (Rasmussen *et al,* 2014).

2.2.2 DLS

Stock concentrations (100mg/L) of NM-300K and NM-104 were made in ultrapure Milli-Q water (18.2 MΩ·cm). Given the highly viscous nature of NM-300K, vials were thoroughly shaken for 5 minutes by hand prior to weighing in volumetric flasks, (via pipette). NM-104 (powder) was weighed directly to volumetric flasks using a spatula. Once NPs had been correctly weighed, volumetric flasks were filled with Milli-Q ultrapure water to give stock concentrations of 100 mg/L and shaken thoroughly by hand before two 15 minute periods of bath sonication (Kerry PUL325). Volumetric flasks were removed from the sonication bath between 15 minute periods and shaken by hand for approximately one minute.

Measurements were made in Milli-Q water and OECD medium (medium) to compare differences in HD and ZP between the original dispersion medium and that used within toxicity testing. OECD medium used was made according to OECD 225 test guidelines, adapted from OECD (1993) as described in Annex A.

Stock solutions of NM-300K and NM-104 were diluted in either Milli-Q water or OECD medium (in 15 ml Fisherbrand® Falcon tubes) to a concentration of 10mg/L. A SRHA stock (50 mg/L in Milli-Q water) was diluted in OECD medium to give a concentratioin

of 5 mg/L SRHA. A stock solutions of NM-300K was then diluted in the SRHA OECD medium to give a concentration of 10 mg/L.

Hydrodynamic diameter (HD) and zeta potential (ZP) measurements were made using the Malvern Zetasizer Nanoseries, Nano ZS. Measurements of HD and ZP were made for the following suspensions across a 96-hour period (0, 24, 48, 72 and 96 hours) in triplicate:

- NM-300K (10 mg/L) in Milli-Q water;
- NM-300K (10 mg/L) in OECD medium;
- NM-300K (10 mg/L) in OECD medium with modified ionic strength (50 and 950 µS/cm to assess extremes of freshwater conductivity which can range between 0-1500 µS/cm (Behar *et al,* 1996);
- NM-300K (10 mg/L) in OECD medium with 5 mg/L Suwannee River Humic Acid (SRHA) – concentration selected based on environmental relevance (Thurman, 1985) and previous work within the research group (John Mullinger and Shona O'Rourke);
- NM-104 (10 mg/L) in Milli-Q water;
- NM-104 (10 mg/L) in OECD medium.

Standard Operating Procedures (SOP) were developed on Malvern software for NM-300K, NM-104 and the media to be used for both NM-300K and NM-104. SOPs ensure that measurements made with the same materials are consistent, whilst speeding up analysis. The zetasizer was turned on 30 minutes prior to analysis to allow the laser to stabilise. A 2 ml syringe was then used to draw up the sample from the first 1-2cm of suspension, (taking care as to limit sample disturbance) and injected into a Folded capillary cell (DTS1060) (ensuring no air bubbles were in the sample) for reading of HD and ZP. The HD and ZP (and PdI) values of samples were recorded in this manner immediately upon their production (time 0), after which they were stored in incubators at 20˚C with a photoperiod of 16:8 light: dark (identical conditions as in exposures) and subsequently measured again after 24, 48, 72 and 96 hours.

2.2.3 DCS

Using the CPS disc centrifuge model DC24000 UHR (at HWU, courtesy of Analytik Ltd.), a centrifuge speed of 23000 RPM was selected for both NM-300K and NM-104 based on particle size and density. Once rotating, a density gradient was built inside the centrifuge disc through the injection of 9 sucrose solutions of decreasing density (24% - 8%) using a syringe. A calibration standard of known size and density (NIST traceable mono-dispersed polystyrene beads) was then injected to calibrate the time axis to particle size. Samples of NM-300K and NM-104 (10mg/L) were prepared in Milli-Q water from 100 mg/L stocks as previously described in section 2.2.1 and (separately) injected (100 µL) into the centrifuge disc. The Disc Centrifuge Control System software (CPS Instruments Inc.) was used to process data.

2.2.4 TEM and EDS

All TEM and EDS work was conducted at Leeds EPSRC Nanoscience and Nanotechnology Facility (LENNF) with assistance from Dr Zabeada Aslam. In total, 8 samples were analysed:

- 10 mg/L NM-300K suspended in Milli-Q water for 2 hours.
- 10 mg/L NM-300K in OECD medium after 2 and 24 hours.
- 10 mg/L NM-300K suspended in OECD medium with 5mg/L SRHA after 2 and 24 hours.
- 10 mg/L NM-104 suspended in Milli-Q water for 2 hours.
- 10 mg/L NM-104 suspended in OECD medium after 2 and 24 hours.

All samples were prepared in 15 ml Falcon tubes (Fisherbrand®) from 100 mg/L NM-300K and NM-104 stocks (prepared and sonicated as described in section 2.2.1) to give final concentrations of 10 mg/L. Following the appropriate time period, 3-4 drops (~15 µL) of each sample were applied to 400 mesh copper grids with holey carbon films (Agar Scientific ®) and left to dry on paper towels under a lamp for 2-3 hours.

Using a CM-200 FEG Philips Transmission Electron Microscope with Oxford Instrument X-Max INCA EDS system, samples were analysed under low magnification (26.5K) to provide images with a sufficient number of NPs to perform size measurements, and

higher magnification (230K) to allow more detailed analysis of morphology. The form factor (measuring particle 'roundness') was calculated by determining the area and perimeter of particles using ImageJ 1.48 software and applying the following formula:

$$Form\ factor = \frac{4\pi \times A}{p^2}$$

Where*: A* = particle area

p = particle perimeter

The resultant values ranged from 0-1, with a value of 1 representing a perfect circle and 0 as an oblong, non-spherical object.

Sections of each micrograph were selected for EDS analysis to determine the elemental composition of the samples, whilst EDS analysis of the TEM grid was performed to confirm elemental composition.

2.2.5 Dissolution (ICP-MS)

All ICP-MS analysis was conducted at the University of Edinburgh with the assistance of Dr Lorna Eades. Dissolution of Ag^+ from NM-300K was investigated for the following suspensions, all at a concentration of 500 µg/L (the LC_{50} value of NM-300K towards *L. variegatus* in OECD medium):

- Milli-Q water (0, 24 and 96 hours);
- OECD medium (0, 24 and 96 hours);
- OECD medium adjusted to 50 µS/cm (0, 24 and 96 hours);
- OECD medium adjusted to 950 µS/cm (0, 24 and 96 hours);
- OECD medium with 5 mg/L SRHA (0, 24 and 96 hours).

A volume of 10 ml of each suspension was made in 15 ml Falcon® tubes (in triplicate) from a 100 mg/L NM-300K stock (prepared as described in 2.2.1) and incubated at 20°C with a photoperiod of 16:8 light: dark. Following the desired time period, suspensions

were transferred to 15ml thickwall polycarbonate centrifuge tubes (Beckman Coulter™) and centrifuged at 25,000 rpm for 1 hour. A volume of 5ml of the supernatant was then transferred to 15 ml Falcon® tubes and acidified with 5 ml of 2% HNO_3 (giving a final concentration of 1% HNO_3). The NM-300K pellet was re-suspended in the remaining 5 ml which was subsequently transferred to 15 ml Falcon® tubes and acidified with 5 ml 100% HNO_3 to give a final concentration of 50% HNO_3 (samples were diluted to 2% HNO_3 (with Milli-Q water prior to sampling with ICP-MS). The sample (1 ml) was transferred to cuvettes and spiked with 10 µL Rhodium (at 2 µg/L) as an internal standard and analysed using an Agilent 7500ce ICP-MS. After every 10 samples, one blank (2% HNO_3) and one 2.5 µg/L Ag sample (PerkinElmer Pure Silver) were run to determine any drift in sensitivity.

2.2.6 Statistical analysis

Data were assessed for normality and homogeneity of variences using the Shapiro-Wilk test. Significant differences between groups (time and media for DLS analysis) were detected via two-way ANOVAs using a (general linear model (GLM). Whenever significant, the pairwise multi-comparison test, Tukey was applied to detect where significant differences existed (i.e. for HD between time points or media). To specifically identify significant differences, individual one-way ANOVA and Tukey tests were performed for media at each time point (IBM SPSS Statistics 22). All TEM images were analysed using ImageJ 1.48 software, with size distribution histograms produced in Microsoft® Excel.

2.3 Results

2.3.1 DLS

Average PdI values for all NM-300K and NM-104 solutions were <0.5 and therefore deemed acceptable for analysis with DLS. All DLS data were found to comply with parametric test requirements. Results from two-way ANOVA analysis confirmed time to have no influence upon the HD of NM-300K, however, a significant difference was detected between media ($p<0.0005$).

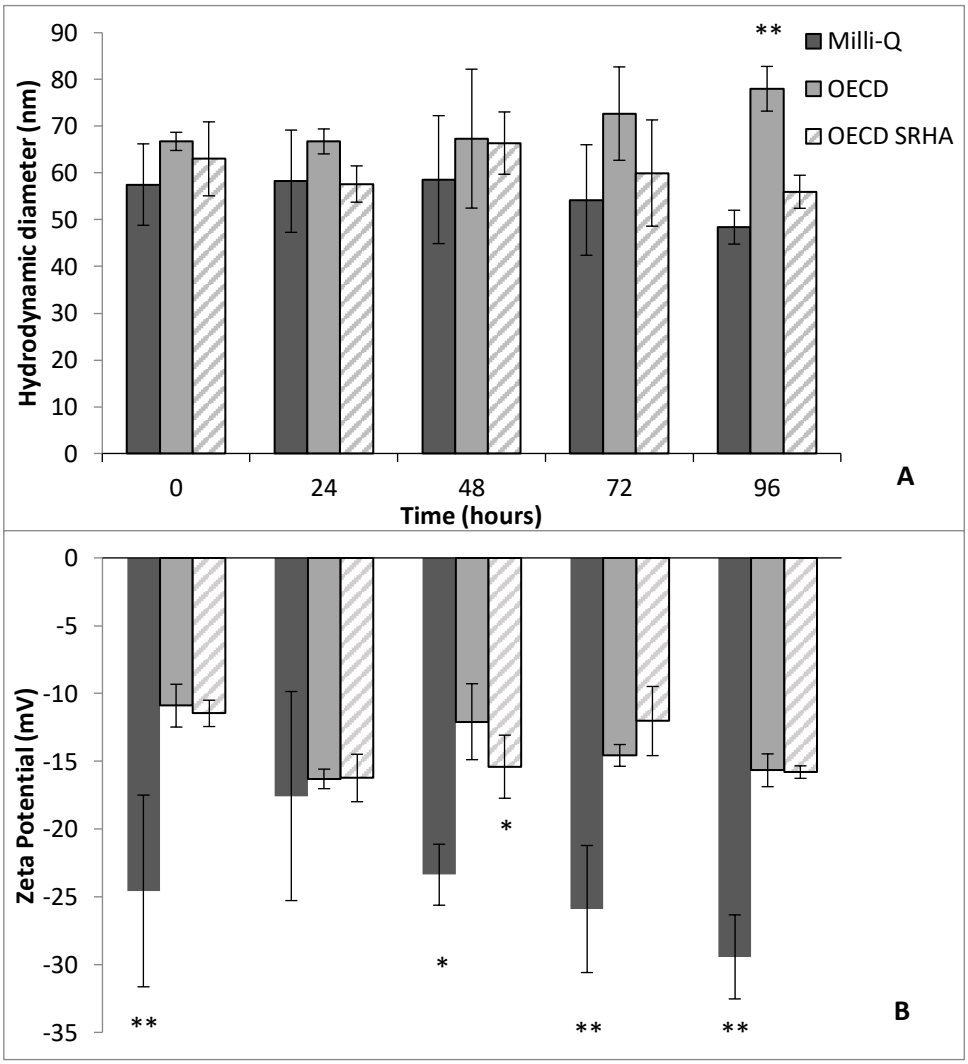

Figure 2.1. Hydrodynamic diameter (A) and zeta potential (B) of 10 mg/L NM-300K suspended in Milli-Q water, OECD medium and OECD medium containing 5 mg/L SRHA for 0-96 hours (data expressed as mean ± SD, n = 3, * = significantly different from one medium within time-point ($p<0.05$), **= significantly different from both media within time-point ($p<0.05$).

Further analysis using Tukey tests confirmed these significant differences ($p<0.05$) to exist between the HD of NM-300K in OECD medium and the HD in both Milli-Q water and 5 mg/L SRHA OECD medium. Following one-way ANOVA and Tukey analysis for 96-hour data, the HD of NM-300K in OECD medium (78 nm) was found to be significantly greater than in Milli-Q water (48.41 nm) and SRHA OECD medium (55.96 nm) (figure 2.1A).

ZP was not influenced by time; however, significant differences were detected between media following two-way ANOVA analysis. Tukey tests confirmed these differences to exist between Milli-Q water and OECD media (with and without SRHA). ZP values for Milli-Q water were considerably more negative, ranging from -17.5 mV to -29.4 mV between 0-96 hours. Individual one-way ANOVA and Tukey tests for data at each time point detected significant differences between Milli-Q water and OECD media ZPs (with and without SRHA), except for 24 hours.

The ionic strength of OECD medium had no significant influence over the HD of NM-300K (0-96 hours), with no difference detected between 50 µS/cm and 950 µS/cm OECD medium (figure 2.2A) and that of standard strength OECD medium represented in figure 2.1 A. Conversely, two-way ANOVA analysis for ZP detected significant differences between the two media. Individual one-way ANOVA and Tukey tests revealed these differences ($p<0.05$) to exist at 24, 72 and 96 hours, with 50 µS/cm medium recording values approximately -21 to -22 mV and 950 µS/cm, approximately -12 to -13 mV) (figure 2.2B). The ZP of NM-300K in standard OECD medium was also significantly less negative than in 50 µS/cm after 0 and 96 hours ($p<0.05$).

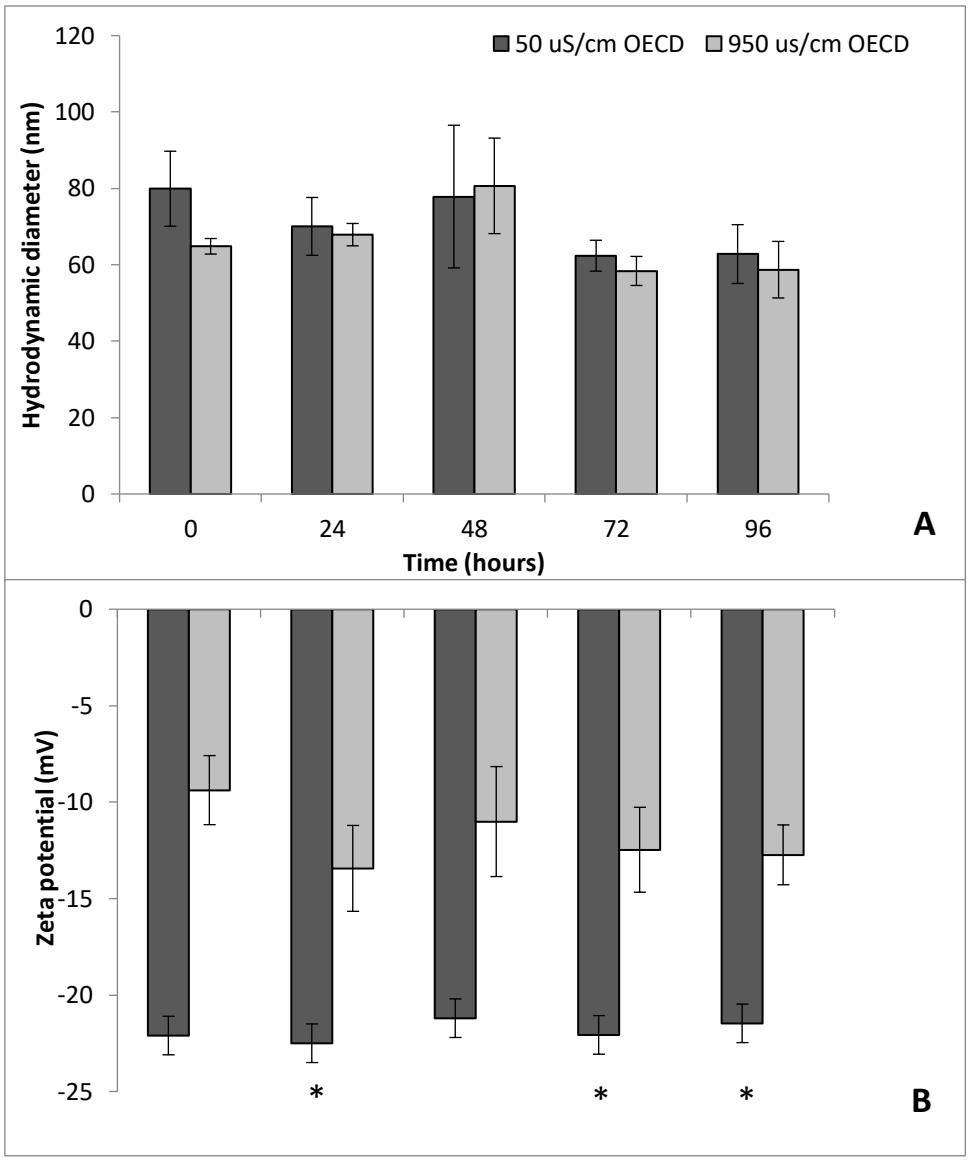

Figure 2.2. Hydrodynamic diameter (A) and zeta potential (B) of 10 mg/L NM-300K suspended in OECD media with ionic strengths of 50 and 950 µS/cm for 0-96 hours (data expressed as mean, n=3, ±SD, *= significantly greater ($p<0.05$).

Significant differences were detected between the HD of NM-104 in Milli-Q water and OECD medium and over the time points tested. Tukey tests detected the differences ($p<0.05$) over time to be between 96 hours and each of the other time points.

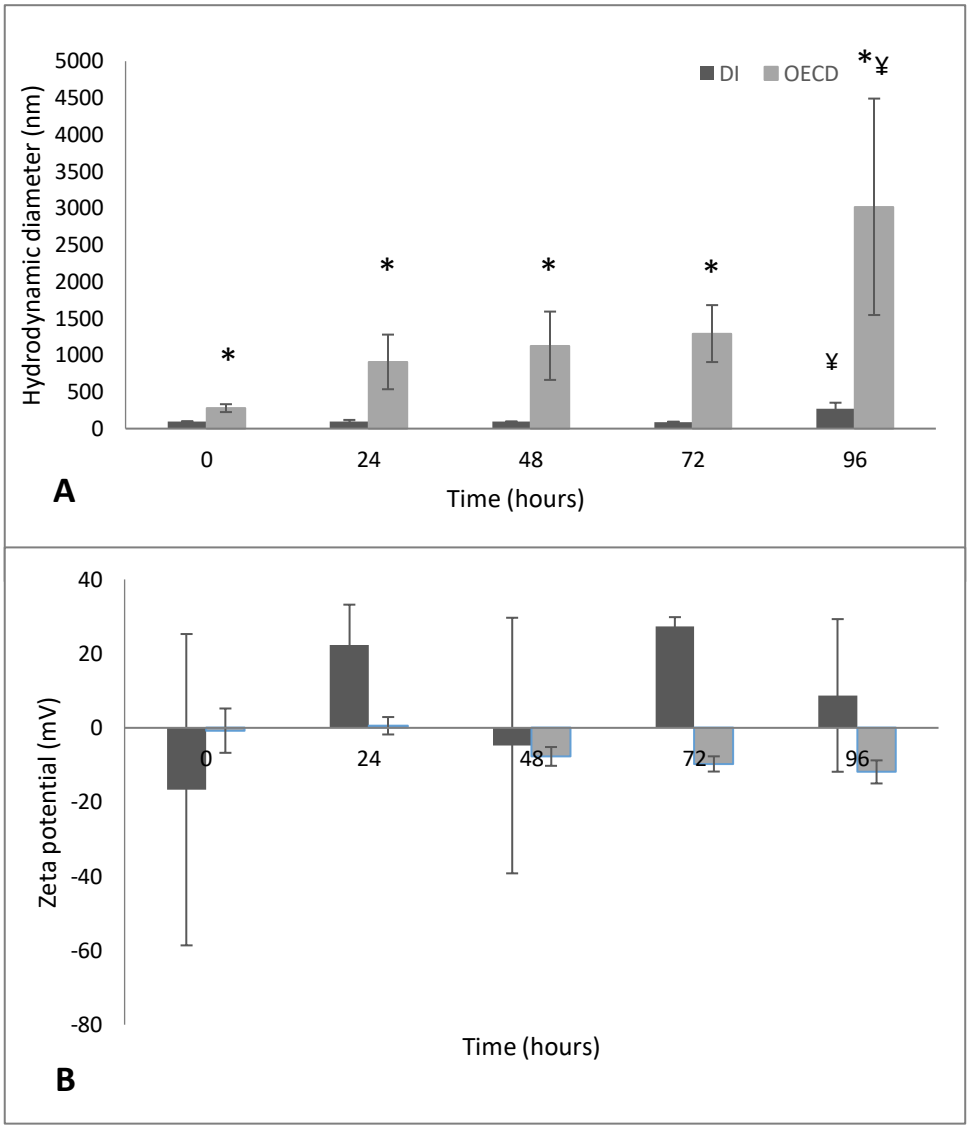

Figure 2.3. Hydrodynamic diameter (A) and zeta potential (B) of 10 mg/L NM-104 suspended in Milli-Q water and OECD medium for 0-96 hours (data expressed as mean, n=3, ±SD, *= significantly greater than Milli-Q water $p<0.05$, ¥ = significantly different from all other time-points, $p<0.05$).

Individual one-way ANOVA and Tukey tests detected significant differences ($p<0.05$) in NM-104 HD in OECD medium and Milli-Q water for each time point – with OECD medium resulting in greater HD values (~280 nm-3000nm) in comparison to Milli-Q (~95-300 nm) (figure 2.3A). ZP was shown to be highly variable for NM-104, particularly in Milli-Q, fluctuating between positive and negative values across the 96-hour period. ZP was more stable in OECD medium, recording negative values at all but one time period (24 hours) (figure 2.3B). Two-way ANOVA analysis revealed medium and time to have no influence upon NM-104 ZP ($p>0.05$).

2.3.2 DCS

A peak of 0.0123 µm (12.3 nm) was detected for NM-300K in Milli-Q water using differential centrifugation sedimentation (figure 2.4), with a mean particle diameter of 15.2 nm (table 2.1). Oversize percentiles (D_{10}, D_{50}, D_{90} - stated in table 2.1) describe the particle size distribution of NM-300K suspended in Milli-Q water. For example, a D_1 value of 34.7 nm (table 2.1), signifies that a 1% mass of NM-300K particles have a diameter of 34.7nm or larger, whilst at D_{99}, 99% mass of particles will have a diameter of 9.8 nm or greater. The D_{50} value is effectively the median value, whereby 50% mass of particles had a particle diameter of 13.5 nm or greater and 50% mass will have a diameter below 13.5 nm.

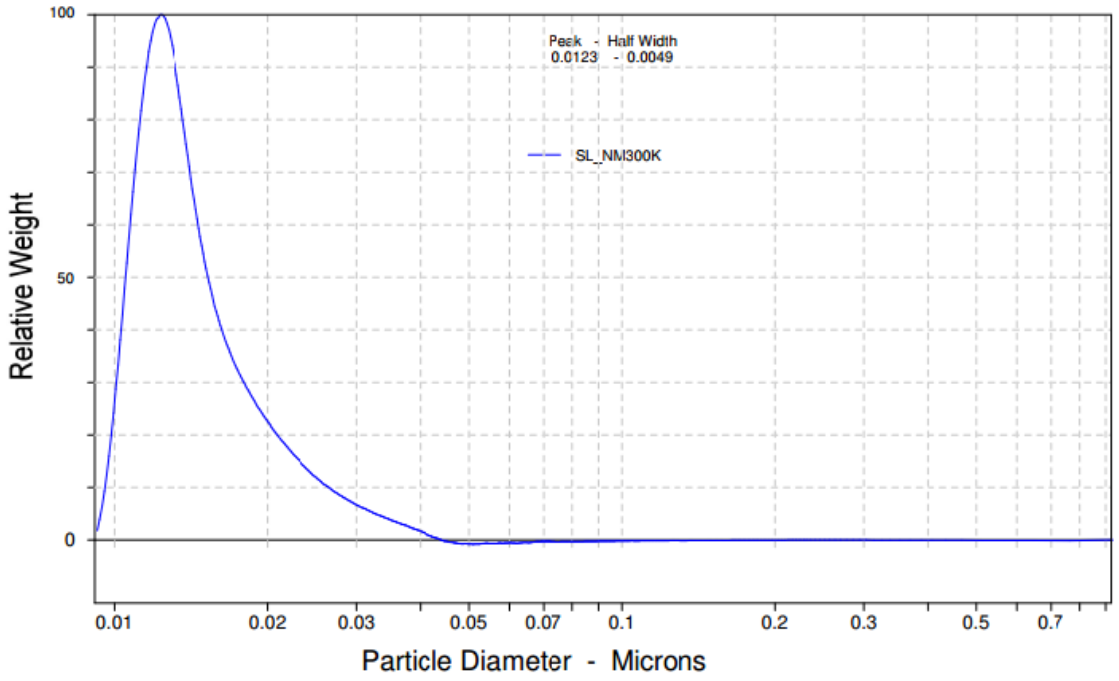

Figure 2.4 CPS Differential Centrifugation Sedimentation data for NM-300K suspended in Milli-Q water n=1.

Unlike NM-300K, where one clear defined peak was detected, DCS measurements for NM-104 detected several wide peaks ranging from 109.5 nm to 294.4 nm (figure 2.5). A mean particle diameter of 221.4 nm was recorded along with D_1 and D_{99} values of 665 nm and 61.5 nm, respectively (table 2.1).

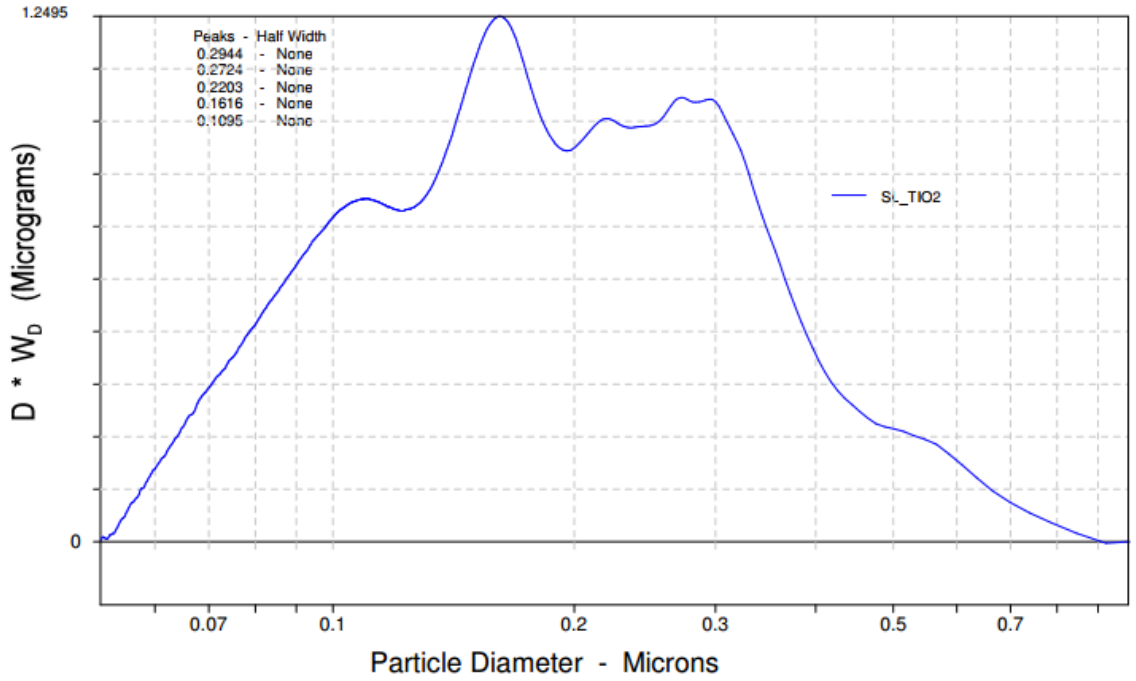

Figure 2.5. CPS Differential Centrifugation Sedimentation data for NM-104 suspended in Milli-Q water n=1.

Table 2.1. DSC detected peaks, mean diameter and oversize percentiles for NM-300K and NM-104 suspended in Milli-Q water.

Particle	Detected peak(s) (nm)	Mean diameter (nm)	D_1 (nm)	D_{10} (nm)	D_{50} (nm)	D_{90} (nm)	D_{99} (nm)
NM-300K	12.3	15.2	34.7	21.8	13.5	10.5	9.8
NM-104	109.5 161.6 220.3 272.4 294.4	221.4	665	389.9	186.4	88.6	61.6

2.3.3. TEM and EDS

TEM image analysis revealed that NM-300K suspended in Milli-Q water recorded a mean particle size of 15.16 nm (table 2.2) with 90% of particles measured being <20 nm.

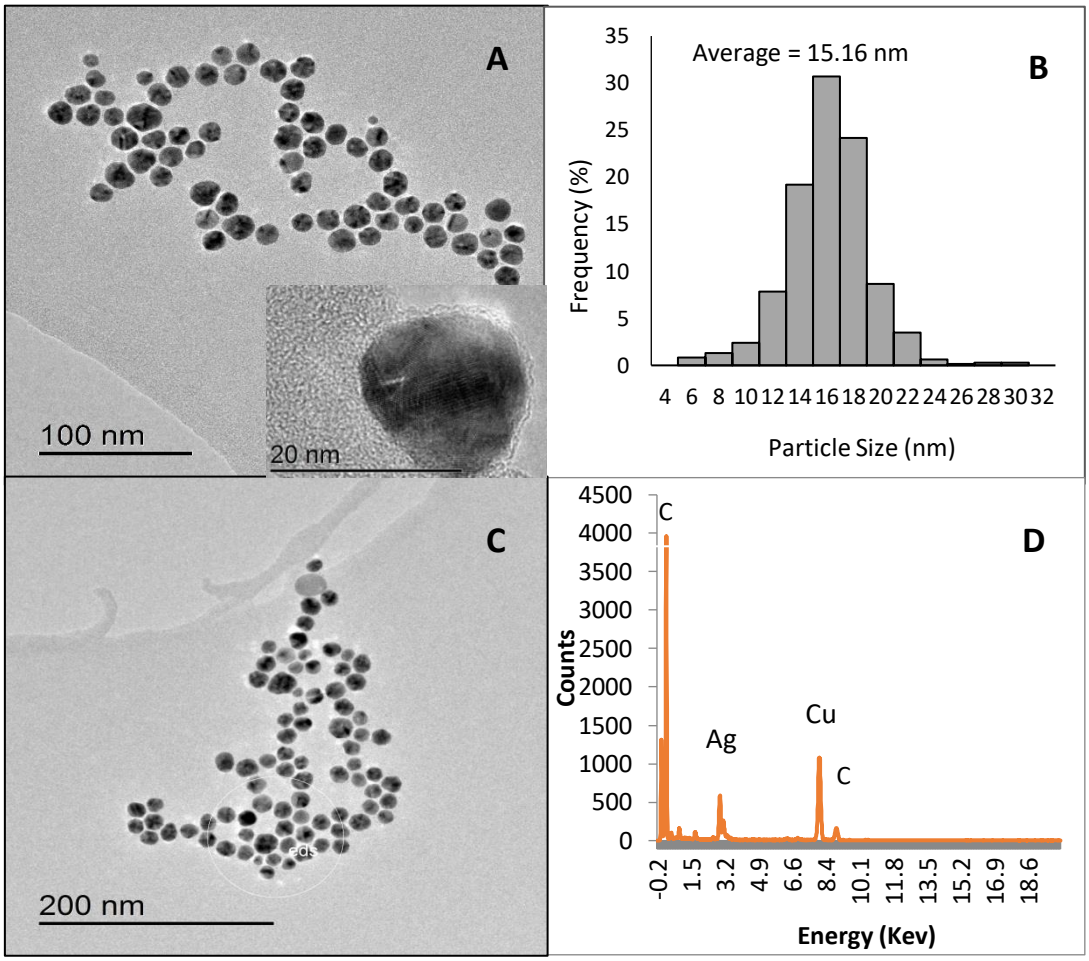

Figure 2.6. A) TEM images of 10 mg/LNM-300K in Milli-Q water for two hours. B) Corresponding size distribution histogram for NM-300K in Milli-Q water. C) TEM image used for EDS analysis. D) Corresponding EDS spectrometer pattern for NM-300K in Milli-Q water.

Although the majority of particles had a somewhat spherical shape – recording a form factor of 0.70 (table 2.2), incidents of triangular or trapezium-like particles were also evident. Higher magnification images (as displayed in figure 2.6A) revealed particles to be crystalline (icosahedron)

When suspended in OECD medium for 2 and 24 hours, NM-300K recorded average particle sizes using of 15 and 16.44 nm, respectively. Although particles were still relatively well dispersed and abundant on TEM grids after 2 hours (figure 2.7A), after 24 hours fewer particles were evident with more evidence of aggregation/agglomeration observed (figure 2.7B). Two-way ANOVA analysis detected significant differences in NM-300K size when suspended in different media ($p<0.001$). Tukey tests detected significantly higher NM-300K sizes when suspended in OECD medium for 24 hours, compared to 2 hours (16.44 nm and 15 nm, respectively) ($p<0.05$). A value of 95.21% of NM-300K particles were <20 nm in OECD medium after 2 hours, whilst 90% of particles were <20 nm after 24 hours.

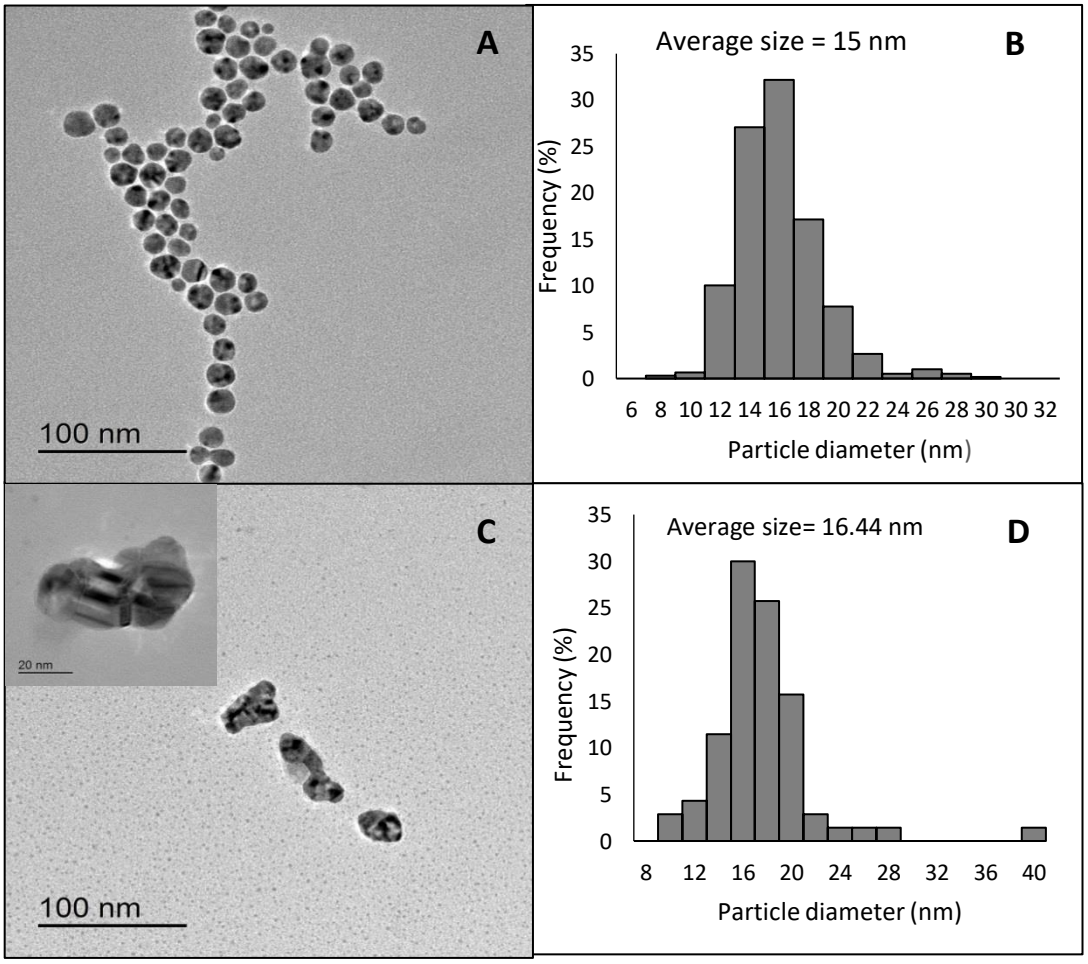

Figure 2.7. A) TEM image of NM-300K suspended in OECD medium for 2 hours. B) Corresponding size distribution histogram. C) TEM images of NM-300K suspended in OECD medium for 24 hours. D) Corresponding size distribution histogram.

Again, NM-300K particles were generally found to be spherical in OECD medium after 2 hours, with a form factor of 0.70 (table 2.2), a value similar to that recorded for NM-300K in Milli-Q water ($p>0.05$). Due to the aggregated state of a number of NM-300K particles in OECD medium after 24 hours (figure 2.7C), it was not possible to generate a form factor value.

Mean particle size for NM-300K suspended in OECD medium with SRHA for 2 hours (16.82 nm, 90% <20 nm) was similar to that of particles suspended in OECD with no SRHA after 2 hours ($p>0.05$) and greater than those suspended in Milli-Q water ($p<0.05$). As with particles in standard OECD medium, after 24 hours in OECD medium with SRHA, fewer NM-300K particles were evident on TEM grids. Tukey tests detected significantly larger particles (average 16.82 nm, 84.6 <20nm) in SRHA OECD after 24 hours in comparison with particles in the same medium after two hours ($p<0.05$). Images captured for NM-300K OECD medium with SRHA generally showed particles to be well dispersed (figures 2.8A & C). However, after 24 hours, there was a tendency of particles to adhere to large filamentous structures (likely components of SRHA) (inset figure 2.8C), whilst some large agglomerate/aggregates were also detected (figure 2.8E) and confirmed to contain silver via EDS (figure 2.8F).

Particles in OECD with SRHA were found to have similar form factors (table 2.2), both of which were found to be significantly greater than particles in all other media ($p<0.05$), indicating greater spherical form.

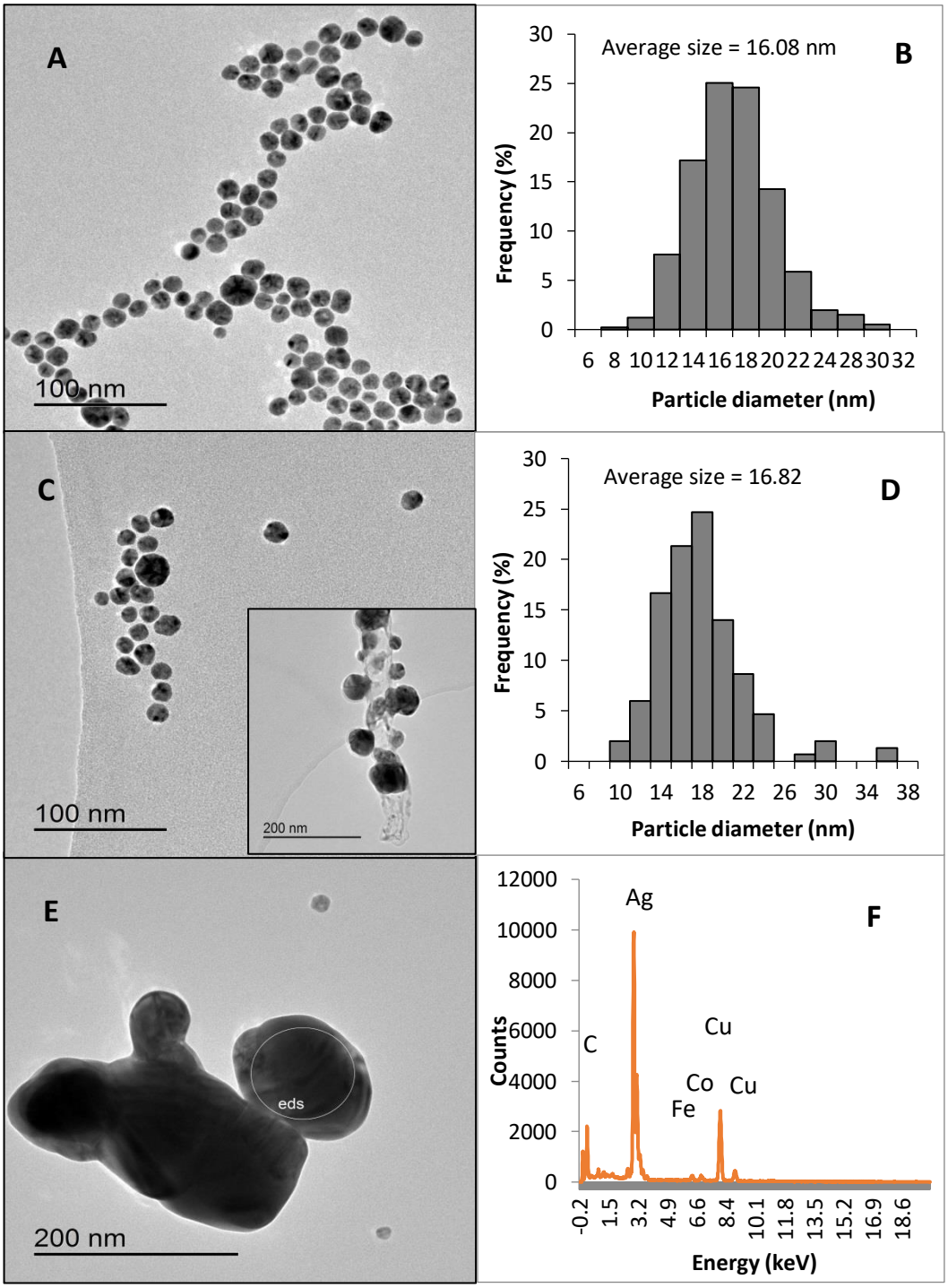

Figure 2.8 A) TEM image of 10 mg/L NM-300K suspended in NM-300K with 5 mg/L SRHA for 2 hours. B) Corresponding size distribution histogram. C) TEM images of NM-300K suspended in OECD medium with 5 mg/L SRHA for 24 hours. D) Corresponding size distribution histogram. E) TEM image of NM-300K agglomerate in OECD medium/ 5mg/L SRHA. F) Corresponding EDS spectrometer pattern

Table 2.2. TEM data for NM-300K (10mg/L) suspended in different media and for different time periods (data expressed as mean, ±SD)

Media	No. ofParticles	Min size (nm)	Max. size (nm)	Mean size (nm)	Form factor
Milli-Q (2 hrs)	625	4.787	29.302	15.16 (±3.06)	0.7 (±0.1)
OECD (2 hrs)	606	7.62	28.29	15.00 (±2.80)	0.7 (±0.12)
OECD (24 hrs)	70	9.77	38.25	16.44 (±4.07)	n/a
OECD w/SRHA (2 hrs)	407	7.92	29.4	16.08 (±3.22)	0.74 (±0.08)
OECD w/SRHA (24 hrs)	150	8.5	35.99	16.82 (±3.93)	0.73 (±0.09)

Due to their highly aggregated/agglomerated state (and likely rapid sedimentation) in OECD medium, even after only 2 hours, it was only possible to record images of NM-104 in Milli-Q water. As evident in figure 2.9A & C, NM-104 particles formed large aggregates and agglomerates, the average size of which was recorded as 159.06 nm (92% <300 nm) (figure 2.9A).

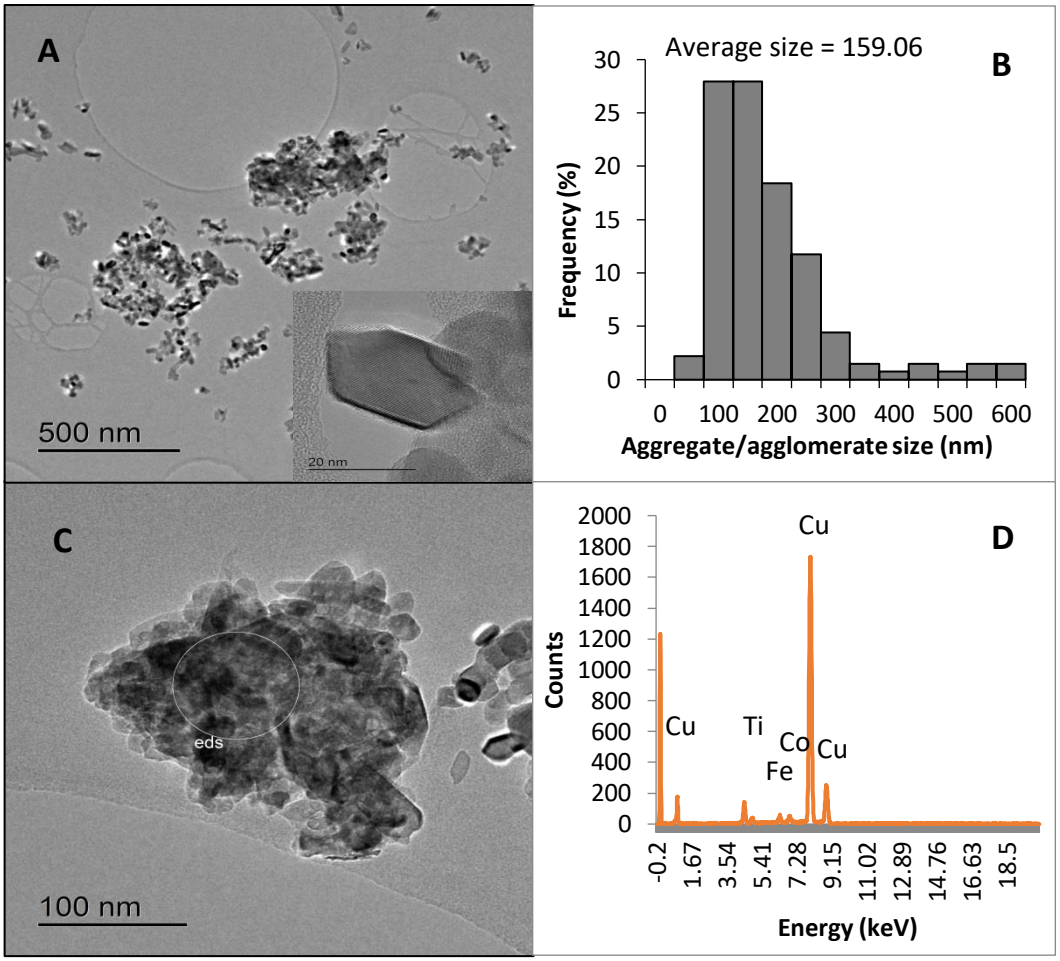

Figure 2.9 A) TEM images of 10 mg/L NM-104 suspended in Milli-Q water for 2 hours. B) Corresponding size distribution histogram. C) TEM image of agglomerated NM-104 particles in Milli-Q water for 2 hours. D) Corresponding EDS spectrometer pattern.

As NM-104 was in a highly-aggregated form, determining a quantitative value for their primary particle size or shape was not possible. Particles generally displayed an elongated, rounded morphology (suggesting an ellipsoidal 3D structure) or more angular elongated morphology as displayed in the inset of figure 2.9A.

Table 2.3. TEM data for NM-104 aggregate/agglomerates in Milli-Q water.

Media	Aggregate/agglomerates measured	Min size (nm)	Max. size (nm)	Mean size (nm)
Milli-Q (2 hrs)	136	39.62	592.8	159.06 (± 106.36)

2.3.4 NM-300K dissolution

Average Ag recovery across dissolution studies (i.e. the combined amount of Ag^+ detected in the digested pellet and supernatant of samples, where the original test concentration - 500µg/L = 100%) was 35.39%. Dissolution values in Milli-Q water after 0 and 96 hours (50.61% and 18% respectively) were found to be significantly greater than dissolution values in all other media at all time points investigated ($p<0.05$) (fig. 2.10).

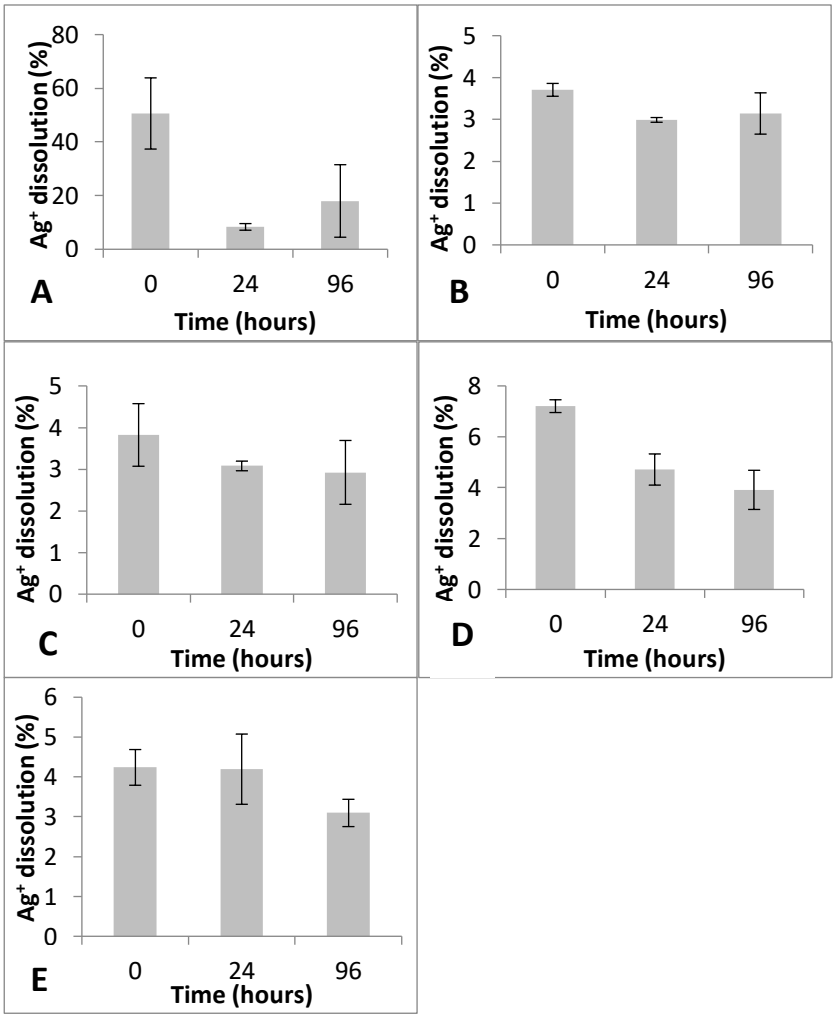

Figure 2.10. Average Ag^+ dissolution from 500 µg/LNM-300K suspended in: Milli-Q water (A); OECD medium (B); OECD medium with 5 mg/L SRHA (C); 50 µS/cm OECD medium (D) and 950 µS/cm OECD medium (E) for 0, 24 and 96 hours (±SD).

Dissolution was unaffected by both OECD medium composition (i.e. change in ionic strength or inclusion of SRHA) and time ($p>0.05$), with values ranging from 2.92% to 7.2% (fig. 2.10).

2.4 Discussion

2.4.1 NM-300K TEM analysis

Whilst TEM images confirmed there is no significant difference in NM-300K particle size when dispersed in Milli-Q water or OECD medium after 2 hours (15.16 and 15 nm, respectively), TEM images in OECD medium after 24 hours showed signs of moderate aggregation/agglomeration (size up to 38.25 nm). Far fewer particles (either aggregated/agglomerated or individual) were evident upon TEM grids for NM-300K in OECD medium at 24 hours (70 compared to 606 after 24 hours). This suggests greater aggregation/agglomeration and potential sedimentation of particles to the bottom of Falcon tubes prior to TEM sampling. In this study, samples for TEM analysis were taken from the mid to upper portion of tubes. Future studies could be used to analyse samples from the bottom portion too, however, could not be included in this study due to the cost and limited availability to TEM. Time related aggregation/agglomeration and sedimentation of NPs within this investigation highlights the importance of sampling across various time-points. In TEM images where aggregation was not prevalent, NM-300K was shown to be relatively polydisperse, with a relatively narrow size distribution (particles ranging from 4.7 nm to 38.25 nm in different media). These findings align with those of Kermanizadeh *et al* (2012) who confirmed NM-300 particles (via TEM) to have a size distribution between 8-47 nm (average 17.5 nm).

TEM analysis of NM-300 (from which NM-300K is sub-sampled - and thereby analogous to) conducted by the JRC confirmed an average particle size of 15 nm, with a narrow size distribution and >99% of particles below 20 nm (Klein *et al,* 2011). Whilst TEM imaging as part of this investigation confirmed similar average size figures, only 90% and 95.21% of particles were found to be <20 nm in Milli-Q water and OECD medium after 2 hours, respectively, indicating a slight deviation from the JRC's findings. Similarly, results obtained via DCS confirmed a comparable average particle size (15.2nm) in Milli-Q water, however, recorded a D_{10} value of 21.8, signifying that 10% mass of NM-300K particles in Milli-Q water were greater than 21.8 nm. Form factor data collected within this investigation confirmed NM-300K to have a shape close to spherical in both Milli-Q water and OECD medium, while some triangular and trapezium-like particles were also evident in TEM images. These findings support those of the JRC (Klein *et al,* 2011)

conducted with NM-300 and Sørensen and Baun (2015) who also cited the occurrence of rod and cubical NM-300K particles amongst predominantly spherical particles.

Although TEM characterisation with more 'pristine' particles, i.e. those suspended in Milli-Q water or OECD medium for a short period of time, produced results similar to those described by the suppliers. However, it is evident that when suspended in OECD medium for a greater length of time (>24 hours), changes to particle properties occurred, most notably in their size and aggregation/agglomeration state. The increasing presence of salts within test medium has shown to induce the aggregation and agglomeration of AgNPs (Römer *et al,* 2013), while polydisperse nanoparticle suspensions (such as NM-300K) are known to settle with time (Witharana *et al,* 2012). ZP results for NM-300K suspended in OECD medium revealed values close to neutral charge, whilst those suspended in Milli-Q water recorded significantly more negative values. Decreased steric repulsion in the presence of salts can lead to aggregation/agglomeration and although no differences in HD were recoded for NM-300K in OECD medium over time, it is likely that aggregation/agglomeration and settling were the cause of fewer particles in TEM images over time – however, further studies would be required to confirm this hypothesis. Digital photography, optical microscopy and Small Angle X-ray scattering (SAXS) have all been used to measure the settling of NPs over time (Witharana *et al,* 2012) and could all be considered to increase understanding of NM-300K settling kinetics.

Alternatively, different TEM preparation techniques could be employed, as both the drying process and solution salt concentration are known to influence NP distribution upon grids (i.e. potentially cause a non-uniform distribution). Repeated sample deposition and drying on grids and sample centrifugation have both been cited as possible techniques to improve TEM images in instances where few particles are evident (nanoComposix, 2012).

2.4.2 NM-300K hydrodynamic diameter

As previously mentioned, no significant increases in average HD were detected over time for NM-300K suspended in OECD medium, however, readings after 96 hours were found to be significantly greater than those obtained for Milli-Q water (78 nm in

comparison with 48.41 nm). Cupi *et al* (2015) found the HD of NM-300K to be considerably greater when suspended in M7 medium in comparison to Milli-Q water (44-63 nm and 347-1316 nm, respectively), with the greatest values recorded for particles aged for 48 hours in M7 medium. Values of HD are typically greater than those recorded via imaging methods as it takes into consideration the electric dipole layer (EDL) of solvents which adhere to a particle when suspended. Consequently, HD readings account for the inorganic core of a particle and its associated hydrated layer, whilst measurements of size by TEM only consider the inorganic core (as the hydrated layer is not visible). The values of HD observed within this investigation generally agree with those determined by the JRC, who cited a mean particle size of 50-70 nm using DLS (Klein *et al,* 2011) and those of Baumann *et al* (2013) who recorded an average HD of 54 nm for NM-300K suspended in Milli-Q water at the same concentration (10 mg/L).

2.4.3 NM-300K zeta potential

Results obtained for ZP indicate that the presence of salts within OECD media reduced the negative charge exhibited by NM-300K particles. Particles were found to be more negatively charged in Milli-Q water (-17mV to -29mV) compared to OECD medium (-10.8mV to -16.3mV). These findings concur with those of Cupi *et al* (2015) who found NM-300K to have a more negative charge when suspended in deioinsed water (ranging from -19 to -22.4 mV across 0-48 hours) compared to when in the OECD medium M7 (ranging from -6.9 to -14.4 mV across 0-48 hours). In the presence of salts, the charge of particles are shielded (in accordance with the DVLO theory), leading to a reduced thickness in the diffuse double layer of NPs EDL (Römer, 2011) – allowing for greater particle-particle interactions (i.e. aggregation and sedimentation). Conversely, when in Milli-Q water, the strong negative charge exhibited by NM-300K allowed repulsion between particles, resulting in well dispersed samples, as observed in TEM images.

Although statistical differences were detected for both the HD and ZP of NM-300K in OECD medium and Milli-Q water, unexpectedly, an increase in the ionic strength of OECD medium only resulted in changes to ZP. The ZP of NM-300K suspended in 50 µS/cm OECD was found to be statistically more negative than NM-300K in standard and 950 µS/cm OECD (whose ZPs were found to be statistically similar), however, no

differences in HD were detected, suggesting that an increase in ionic strength did not cause NM-300K aggregation. These findings go against those of Bradway *et al* (2010), Stebounova *et al* (2011) and Delay *et al* (2011) who found the aggregation of AgNPs (with various coatings) to increase following an increase in ionic strength. Although increased NM-300K aggregation was not witnessed in increasing ionic strength in this investigation via DLS, it is possible that aggregates may have settled and gone undetected. By complimenting DLS with centrifugal particle sizing (as performed for NM-300K in Milli-Q water), a better understanding of the aggregation state of NM-300K in OECD medium (of various ionic strengths) could be achieved.

2.4.4 NM-300K in SRHA OECD medium

The coating of NPs with humic acids typically creates a negative surface charge, increasing steric repulsion and thereby, NP stabilisation. The addition of an organic source to OECD medium, in the form of SRHA, was shown to have a relatively stabilising effect on NM-300K, with more particles evident on TEM grids after 24 hours, suggesting less agglomeration/aggregation and sedimentation, whilst particles displayed greater dispersion. Particles also recorded a larger average size when suspended in SRHA (16.08 and 16.82 nm after 2 and 24 hours, respectively). An increase in size could be attributed to a SRHA coating of particles, however no such coating was readily observable in higher magnification images, although changes in the composition of ecotoxicological media have previously shown to affect NP size (Römer *et al,* 2011; Tejamaya *et al,* 2012).

Despite an apparent particle stabilisation, the ZP of NM-300K was unaffected by the inclusion of SRHA in OECD medium, being significantly similar to that of NM-300K in standard OECD and significantly less negative than NM-300K in Milli-Q water. These results agree with those of Cupi *et al* (2015) who, after suspending NM-300K in M7 medium with and without SRHA (20 mg/L) detected no differences in ZP after 48 hours. However, Cupi *et al* (2015) observed no stabilising effect of SRHA upon NM-300K, with large aggregates (micrometer range) detected after 48 hours, citing the high ionic strength of M7 medium as a likely cause. Conversely, HD data (supported by TEM analysis) within this investigation, confirmed SRHA to have a stabilising effect upon NM-300K after 96 hours, with statistically lower values recorded in comparison with OECD

medium containing no SRHA (55.9 nm compared to 78 nm). These findings agree with those of Fabrega *et al* (2009), who found SRHA (10 mg/L) to stabilise NM-300K and cause partial disaggregation.

Although a considerably greater SRHA concentration (20 mg/L) was used within the work of Cupi *et al* (2015) – which has shown to increase AgNP aggregation/agglomeration in other studies (Gao *et al,* 2012), it is likely that differences in media composition influenced NM-300K stability in the presence of SRHA, highlighting the importance of NP characterisation within test media. A concentration of 5 mg/L SRHA was selected within this investigation to represent an environmentally relevant NOM level, however, it is possible that changes in NM-300K charge may have been observed using a greater SRHA concentration and could be considered in future NM-300K-SRHA investigations.

Large filamentous structures evident in TEM images for OECD medium containing SRHA were likely to be fragments of decomposing vegetation (from which SRHA is predominantly composed), and clearly interacted with particles. Large dark material evident on TEM grids were also presumed to be a component of SRHA and following EDS analysis, was shown to contain NM-300K. Incidents of NM-300K adhesion onto and incorporation into SRHA components is likely to influence their fate, bioavailability and toxicity towards test species.

2.4.5 Dissolution

Although intrinsic physicochemical properties of NPs can often influence dissolution, so too can the media in which they are suspended. As previously discussed, changes in pH, ionic strength and the introduction of NOM can influence NP stability within environmental media. Increased aggregation or dispersion because of media composition will determine the surface area of NPs which are exposed and thus influence the rate of dissolution.

Dissolution in Milli-Q water after 0 and 96 hours was significantly higher than in any of the OECD media tested at any time point. NM-300K has previously shown to display greater dispersity in Milli-Q water (greater negative charge) and have a lower HD,

indicating a thinner electric dipole layer. Both factors limit aggregation, increasing the surface area of particles that are exposed and thus provide a logical explanation for their increased dissolution observed. Conversely, the inclusion of salts in OECD medium, (which reduced ZP and increased HD of NM-300K during DLS studies) limited dissolution in comparison, with increased aggregation/agglomeratioin and steric protection likely explanations.

Reducing the ionic strength of OECD medium (which was found to cause a more negative ZP value for NM-300K) had no significant influence upon dissolution, despite recording a relatively higher value after 0 hours. Similarly, an increase in ionic strength (950 µS/cm) induced no significant deviations in dissolution from those obtained for standard OECD medium. Studies have demonstrated the ability of humic acids to either enhance or inhibit dissolution via ligand promotion or steric protection, respectively (Furrer *et al,* 1986; Yoon *et al,* 2005). Within this investigation, SRHA had no significant effect upon the dissolution of Ag^+ from NM-300K, coinciding with the results obtained from DLS. Again, (despite being environmentally relevant) the concentration of SRHA used may have been too low to have any significant impact upon NP behaviour.

Time had no effect upon dissolution, with no statistical differences detected between samples taken at 0, 24 or 96 hours, indicating that the majority of Ag^+ dissolution from NM-300K occurs rapidly once introduced into testing media. Within the literature, dissolution of Ag^+ from AgNPs is generally considered to be relatively high, given the correct circumstances (Liu *et al,* 2010) and is thought to be the primary mode through which they exert toxicity (Yang *et al,* 2011). Dissolution of Ag from NM-300K within this investigation was found to be relatively low (3-4% across all time periods tested), potentially limiting Ag^+ modes of toxicity. Although total silver recovery was poor, the values obtained for the dissolved fraction were within the range anticipated, based on the findings of similar dissolution studies conducted with NM-300K. Cupi *et al* (2015) also reported values of 3-4% dissolution, while Mallevre *et al* (2014) reported dissolved fractions between 1.83-3.88% in artificial water and Luria Bertani medium. Additionally, Kermanizadeh *et al* (2012) detected very low rates of dissolution (<1%) for NM-300 in pure water and C3A complete medium.

Poor recovery of total silver in NM-300K dissolution studies was likely due to the highly adsorbent nature of Ag. Recovery efficiency of AgNP during dissolution studies is known

to be compromised by the adsorption of silver to apparatus used within separation stages (Misra *et al,* 2012). Within this investigation, a relatively low NM-300K concentration (500 μg/L) was used to reflect actual concentrations used with toxicity testing (LC_{50} value recorded in chapter 3). Consequently, any small losses of Ag had greater repercussions than if a much larger concentration had been used. It is probable that following ultracentrifugation, silver within the 'particle' fraction of samples became adsorbed to ultracentrifugation tubes and lost during the dilution phase. Cupi *et al* (2015) also experienced poor recovery (54-85%) of NM-300K during dissolution experiments, also hypothesising NM-300K sorption to glass and subsequent losses in serial dilution stages as a likely cause, rather than difficulties in analytical and chemical analysis. The technique of centrifugal ultrafiltration is increasingly being used as an efficient separation procedure with good rates of recovery (Misra *et al,* 2012) and may be a possible avenue to explore in future NM-300K dissolution experiments.

2.4.6. NM-104 TEM analysis

NM-104 readily formed large aggregates/agglomerates almost immediately upon their introduction to Milli-Q water and OECD medium as confirmed by both DLS and TEM. Aggregation/agglomeration occurred to a greater extent in OECD medium. As previously hypothesised for NM-300K, NM-104 aggregation/ agglomeration in OECD medium led to their settlement in test vessels and consequently, no particles were evident on TEM grids even after a period of only 2 hours (samples for TEM were taken from the mid to upper portion of tubes). The settlement of NM-104 has previously been studied by Tavares *et al* (2014) who found ca. 60% of particles to settle (in RPMI 1640 cell culture medium) after 6 hours using measurements of zeta potential. Images in Milli-Q water after 2 hours revealed an average aggregate/agglomerate size of 159 nm, while DCS confirmed an average size of 221.4 nm.

2.4.7 NM-104 hydrodynamic diameter

Measurements of HD also confirmed the formation of NM-104 aggregates/agglomerates upon their introduction to Milli-Q water and more profoundly in OECD medium, with values ranging from ~300-3000 nm across 0-96 hours. Characterisation conducted by the JRC (Rasmussen *et al,* 2014) confirmed that NM-104 strongly aggregated/agglomerated within Milli-Q water with only a few single particles observed using TEM. Rasmussen *et al,* (2014) also observed agglomerates/aggregates to ranged between 20nm and 500nm in Milli-Q water with an average diameter of 117.8nm. Although Nischwitz and Goenaga-Infante (2012) confirmed NM-104 to have a primary crystal size of 20 nm (using X-ray diffraction), they found it to form large aggregates (size not stated) when in suspension (water and water with methanol).

Faria *et al* (2014) recorded a mean HD of 1361.33 nm for NM-104 suspended in fish medium (reverse-osmosis purified water) for 24 hours, while Nicolas *et al* (2015) observed a rapid aggregation/agglomeration upon introduction to OECD algal growth medium (1224 nm after 10 minutes). Similarly, Campos *et al* (2013) recorded NM-104 primary particle size to be 20nm (via TEM) but confirmed aggregates/agglomerates to have an average hydrodynamic size of 699 nm in hard synthetic water (at a concentration of 10mg/L) after 24 hours. Using the same concentration, a comparable HD (907.9 nm) was recorded for NM-104 in OECD medium after 24 hours, further confirming the tendency of the particles to aggregate/agglomerate.

2.4.8 NM-104 zeta potential

The tendency for NM-104 to agglomerate/aggregate is most likely related to surface charge. As previously discussed, the introduction of salts generally reduces the ZP of particles, limiting their steric repulsion and thus increasing the formation of aggregates and agglomerates. DLS confirmed NM-104 to have a relatively neutral ZP in OECD medium, particularly after their initial introduction (0 hours). These values became increasingly more negative after 24 hours, reaching a value of -11.87 mV after 96 hours; however, it is highly likely that NM-104 particles were already in a highly agglomerated/aggregated state at this time point. Within Milli-Q water, the ZP of NM-104 fluctuated between relatively positively and negatively charged values, improving

steric repulsion between particles. Hammond *et al* (2013) and Nicolas *et al* (2015) also found NM-104 to have a greater charge in Milli-Q water (34.59 mV and 23mV, respectively) than when suspended in media (7.65 mV in L-15 medium and 12 mV in OECD algal growth medium, respectively).

The stability of TiO_2NPs, including NM-104, is known to be greatly influenced by the pH of suspension media. Agglomeration/aggregation is generally greatest when pH nears the point of zero charge (pH_{zpc}) due to a decrease in electrorepulsion between particles (Sharma, 2009), whilst there is evidence to suggest that an increase in TiO_2NP size increases the value of pH_{zpc} (Sharma, 2009). The JRC (Rasmussen *et al,* 2014) found NM-104 to form stable suspensions at a pH 4 and below and cited a pH_{zpc} value of 8.2 (accrediting the high value to the presence of Al on the surface coating on NM-104). With a pH of ~7.7, OECD medium is near to the pH_{zpc} for NM-104, which goes towards explaining the high degree of aggregation witnessed. Although changes to media composition (i.e. pH) were not considered in relation to NM-104 within this project, it is probable that the stability and mobility of NM-104 would increase across the pH range (and away from the pH_{zpc}).

2.5 Conclusion

This investigation highlights the importance of NP characterisation, both to confirm NP properties and to assess their behaviour within ecotoxicological test media. Although NM-300K characterisation in Milli-Q water provided comparable results to those of the manufacturer (i.e. average size and shape), differences did exist between size distribution data (i.e. 99% of particles were not found to be <20nm) – however, it is possible that these discrepancies could be related to methodologies used within TEM analysis.

While NM-300K formed stable, well dispersed suspensions in Milli-Q water, aggregation/agglomeration was evident in OECD medium (confirmed in TEM images and DLS data). Such changes to NM-300K properties (HD, ZP, aggregation/agglomeration) highlight the importance of NP characterisation in environmentally relevant test medium. The aggregation/agglomeration of NM-300K is likely to affect their fate, behaviour and toxicity in test scenarios (e.g. towards *L. variegatus* within this

investigation) and upon their release into the environment. NM-300K dissolution was found to be low, which is in accordance with other available research conducted with NM-300K. Using the dissolution data collected within this chapter, it is possible to advise whether potential toxicity towards *L. variegatus* is due to the release of silver ions (known to be highly toxic) or as a direct result of NM-300K (i.e. nano-specific toxicity).

Across the available literature, NM-104 is widely accepted to form large aggregates/agglomerates – as observed within this investigation. Considerably larger aggregates/agglomerates were observed in OECD medium, which is likely to have an inhibitory effect upon any potential nano-specific modes of toxicity towards environmental test species such as *L. variegatus*.

The use of complimentary characterisation techniques proved to be highly beneficial within this study, however, further optimisation of certain techniques (e.g.TEM imaging of NPs in test media and NM-300K dissolution) may provide a more quantitative insight and is something that should be considered in further studies.

Chapter 3: Acute aquatic toxicity testing and the influence of abiotic factors towards *L. variegatus* survival and behaviour.

3.1 Introduction.

Abiotic conditions of freshwater environments are likely to determine the phase in which nanoparticles will reside, i.e. water column or sediment (Keller *et al,* 2010) and their bioavailability to aquatic and benthic organisms. For example, the agglomeration/aggregation and colloidal stability of NPs are likely to be largely determined by the combined effects of environmental pH, ionic strength, natural organic matter (NOM) and temperature. Although studies have investigated NP agglomeration/aggregation in complex aqueous matrices (Keller *et al,* 2010; Römer *et al,* 2011), research regarding the direct influence of abiotic conditions to freshwater NP toxicity is still limited. *L. variegtus* occupy a wide range of environments both at the sediment/water and water/air interface (Drewes, 1997), and consequently experience several biological and chemical gradients, making them highly desirable for the environmentally relevant testing of NPs, particularly given the tendency for NMs to settle in aquatic systems. The distinctive and well defined behavioural traits of *L. variegatus* also provide a useful and easily measured insight into sub-lethal toxicity.

3.1.1 Influence of abiotic factors on NP fate and behaviour

Understanding the aggregation/agglomeration, sedimentation and dissolution of NPs is vital when considering their fate and behaviour in the environment. Although the aforementioned processes are highly dependent upon NP properties (as investigated in the previous chapter), they are also strongly influenced by abiotic factors such as pH, ionic strength and natural organic matter content. For example, when NPs experience a pH value within their point of zero charge (PZC) - whereby particles exhibit a net proton charge of zero, their electrostatic forces are reduced, promoting NP aggregation/agglomeration (Umh and Kim, 2014). Conversely, in conditions where the pH is outside the PZC for a particular NP (PZC is material specific), particles exhibit greater repulsive forces, limiting aggregation/agglomeration (Baalousha *et al*, 2009).

The balance between attractive van der Waals and repulsive electrical double layer (EDL) forces are the basis of the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, which dictates NP aggregation/agglomeration in the aquatic environment (Boström *et al,* 2001) which ultimately has a large bearing on toxicity.

The EDL charge of NPs not only reflects that of the NP surface, but also the solution chemistry of the surrounding medium (Hotze *et al,* 2010). The quantity of ions within a given medium (i.e. ionic strength) determines the radius of the diffuse layers from the surface of the particle. Under low ionic strength conditions, the diffuse layer is allowed to extend, whilst under increasing ionic strength the EDL is compressed, limiting steric forces and electrostatic repulsion between two particles with the same charge (Navarro *et al,* 2008). Under such circumstances, the probability of NP aggregation/agglomeration is greatly enhanced.

The interaction of NPs with NOM, which is ubiquitous throughout the aquatic environment, also influences their surface charge and consequently, toxicity. The composition and concentration of NOM in freshwater systems varies according to location, however, humic acids are generally considered one of the main constituents (Frimmel, 1998). The sorption of organic matter by NPs through, electrostatic and hydrophobic interactions and hydrogen bonding (Hyung *et al,* 2007), alters stability in the process of steric interactions. When adsorbed NOM layers occur, nanoparticles develop a negative charge irrespective of their initial surface charge (Amirbahman and Olson, 1993) and thus repel one another to a greater extent (Zhang *et al,* 2009). Increased steric or electrostatic repulsion increases the stability of nanoparticles in natural aquatic environments, whilst destabilisation only occurs when surface charge is nearly neutralised (Navarro *et al,* 2008). Should destabilising conditions prevail (i.e. low organic matter content), nanoparticles are more likely to settle out of the water column and become deposited in benthic systems, including sediments (Stankus *et al,* 2010).

3.1.2 Lumbriculus variegatus behaviour

Understanding the relationship between abiotic factors and NP toxicity is imperative when considering their ecotoxicological effects. *L. variegatus* inhabit a wide range of freshwater environments across Europe and North America, where abiotic conditions will undoubtedly vary. Although studies of mortality following NP exposure provide imperative data within ecotoxicological research, it is likely that *L. variegatus* and other environmental species will encounter NPs at sub-lethal concentrations. Consequently, the study of discreet, sub-lethal responses can offer a greater insight into NP toxicity.

L. variegatus are amongst one of the few oligochaete species capable of swimming and represent the only Lumbriculidae in which such behaviour has been studied in detail (Drewes, 1999). Distinctive behavioural traits are mediated by the medial (MGF) and two lateral (LGF) giant fibres located in the ventral nerve cord of *L. variegatus*. Each of the giant fibres originates from enlarged axons arising from segmentally arranged interneurons (Drewes, unpublished). Touch sensory stimulation of anterior *L. variegatus* segments excites *L. variegatus* MGF, whilst identical stimulation (in addition to shadow stimulus) of posterior segments excites the two LGFs. Once excited, both MGF and LGF conduct impulses along the ventral nerve cord, exciting segmental motor neurons which subsequently activate longitudinal wall muscles. This, in turn, results in the rapid shortening and withdrawal of both anterior and posterior segments (Drewes, 2002).

Drewes (1999) observed body reversal behaviours of *L. variegatus* following tactile stimulation of their 30% most anterior segments. Following an initial end-to-end body shortening, Drewes describe the simultaneous bending of head, body and tail segments to form an '*omega shape'*. The bending of tail segments continues in a wave-like motion towards the original anterior position, during which, the body and anterior segments begin to straighten to form a loop. Further tail progression and body straightening ultimately initiate the loop opening and completion of body reversal (figure 3.1). Drewes (1999) recorded body reversal to take approximately 0.6 seconds for adult *L. variegatus,* with a 140-150°change in the longitudinal axis and minimal translocation in the central point.

Figure 3.1. Silhouette, freeze-frame images of the stages of *L. variegatus* body reversal following tactile stimulation of head segments. Source: Drewes, 1999.

Conversely, light tactile stimulation to the posterior half of *L. variegatus* has shown to evoke swimming responses. Rapid waves of helical bending originate from the anterior end and rapidly progress towards the posterior, propelling the worm forward in the process. Detailed analysis from Drewes (1999) showed waves to alternate from clockwise to anticlockwise helical orientation, giving the impression that *L. variegatus* corkscrew through the water (O'Gara *et al,* 2004). Although the frequencies of helical waves appear to vary between *L. variegatus* at different developmental stages, each swimming occurrence is thought to last between 1-3 seconds (Drewes, 1999).

The behavioural responses described are only exhibited in open water and exclusively upon tactile stimulation, suggesting that they are specifically designed to evade predation. In such a scenario, it is proposed that physical attack to *L. variegatus* anterior sections stimulates body reversal, allowing helical swimming away from predation following further stimulation to the posterior. Diminished or complete loss of *L. variegatus* behavioural responses because of contaminant exposure could potentially increase predation and could lead to greater ecological repercussions. Although locomotive responses of *L. variegatus* have been used sparingly within ecotoxicological research, O'Rourke *et al* (2015) reported behavioural assays to be a suitable technique for the assessment of the acute hazards associated with NP exposure.

3.1.3 Aims

The aims of this chapter were to assess the acute toxicity of NM-300K (silver) and NM-104 (titanium dioxide) to *L. variegatus* in 96 hour, water-only exposures via assessment of mortality and behavioural responses (body reversal and swimming). In addition, the influence of abiotic factors; pH, ionic strength and NOM content on NP toxicity were investigated. The toxicity of silver nitrate ($AgNO_3$) was also examined to compare the toxicity between silver in salt and nanoparticle form.

3.2 Methods

3.2.1 OECD medium.

OECD medium (as described in chapter 2) was used for all experiments and the laboratory culturing of worms.

3.2.2 L. variegatus culturing

All experiments were performed using *L. variegatus* cultured in small aquaria with approximately 5 litres of OECD medium. Torn strips of washed and autoclaved paper towel were used as a fibrous substrate of decomposing material for both the worms and any microscopic organisms (e.g. bacteria, protozoans, rotifers, or ostracods) occupying the culture. Cultures were kept in a temperature controlled incubator at 20ºC with a photoperiod of 16:8 (light: dark), gently aerated, and fed powdered fish food (1g) twice a week. The tank was cleaned (using tap water, then rinsed with Milli-Q water) on a weekly basis, during which OECD water was replaced. Paper towels were replaced on a biweekly basis.

3.2.3 L. variegatus synchronisation

To ensure physiologically synchronised populations for use in experiments, healthy adult worms were transferred to a petri dish containing a small amount of OECD medium (via a plastic Pasteur pipette) and, using a scalpel, cut just below the head segment. Fragmented head and tail segments were transferred to separate beakers (1L) containing approximately 700 ml OECD medium and paper towels and stored for 12-14 days in the conditions described in section 3.2.2, after which they could be used in the experiments. Worms were fed powdered fish food following 7 days into the regeneration process.

3.2.4 Nanoparticle preparation

Stock suspensions of NM-300K (100 mg/L) and NM-104 were prepared as described in chapter 2 (section 2.2.1)

3.2.5 L. variegatus nanoparticle exposure

Due to the lack of relevant data in the literature, range-finding pilot studies were conducted for the toxicity of both NM-300K and NM-104 towards *L. variegatus.* Concentrations of; 0.01, 0.1, 1 and 10 mg/L and 25, 50, 75, 100 mg/L were selected for NM-300K and NM-104, respectively (a lower silver concentration was selected as silver is generally regarded to be more toxic than titanium dioxide). Serial dilutions were made from NP stocks (100 mg/L and 1000 mg/L for NM-300K and NM-104, respectively) of which 20ml were distributed to disposable plastic scintillation vials. Five vials were used per NP concentration, in addition to a control (OECD medium only). To each vial, one healthy, synchronised *L. variegatus* was transferred using a wooden pick (so as not to introduce additional medium). Vials were stored in the conditions described in section 3.2.2, without food. Following 96 hours of exposure, worm mortality and behaviour (as described in section 3.2.9) were assessed. Worms were considered dead if any of the following applied:

- No movement upon mechanical stimulation;
- Decomposed remains evident;
- No worm visible (i.e. complete decomposition).

NM-300K pilot studies allowed full toxicity testing experiments to be designed. Concentrations of 0, 0.125, 0.2, 0.25, 0.4, 0.5, 0.6, 0.75, 1, 1.25 and 1.5 mg/L were tested. Full toxicity testing was performed as described for pilot studies, however using 10 worms per concentration and repeating exposures 3 times. Toxicity tests were also conducted with NM-300K DIS (dispersant of NM-300K) to assess whether it accounted for any of the toxicity measured. Worms were exposed to NM-300K DIS at a weight equivalent to 1.5 mg/L NM-300K (the highest NM-300K concentration tested) for 96 hours as described above.

3.2.6 L. variegatus silver nitrate exposure

Silver nitrate ($AgNO_3$) toxicity studies were initially conducted using a concentration range similar to that previously used in NM-300K testing, (0.25-1 mg/L). Subsequent toxicity testing using an amended concentration range (0, 10, 20, 30, 40, 50, 100, 125,

150 and 175 µg/L) was performed as described in section 3.2.5 using an $AgNO_3$ stock of 100 mg/L ($AgNO_3$ weighed into a volumetric flask and topped up with Milli-Q water).

3.2.7 Assessing the effect of natural organic matter content

Suwannee River Humic Acid Standard II (SRHA), purchased from International Humic Substances Society (IHSS) was used for all NOM experiments. Initial experiments were used to investigate the effect of a range of organic matter concentrations on NM-300K toxicity towards *L. variegatus.* A 500 mg/L SRHA stock concentration was prepared by weighing SRHA directly into a volumetric flask, filling with Milli-Q water (100 ml) and stirring overnight to assure full dissolution. Ten-fold dilutions were then made in OECD medium to achieve SRHA concentrations of 0.5, 5 and 50 mg/L (all considered to be environmentally relevant levels). In this pilot study, a concentration range of 0, 0.125, 0.25, 0.5, 0.75, 1, 1.25 and 1.5 mg/L NM-300K was prepared as described in section 3.2.5, using one replicate. Vials were stored for 96 hours under the conditions described in section 3.2.2, after which, mortality was assessed in accordance with section 3.2.5.

Subsequently, worms were exposed to 5 mg/L SRHA conditions using a concentration range of 0, 0.125, 0.25, 0.4, 0.5, 0.6, 0.75, 1, 1.25 and 1.5 mg/L.

3.2.8 Assessing the effect of pH and ionic strength

Pilot studies to investigate the impact of pH on *L. variegatus* mortality were conducted using 3.5 mM MOPS buffer (3-(N-morpholino)propanesulfonic acid) and 1M NaOH to alter the pH of OECD medium (typically ~pH 7.7) to 6.5 and 8.5. Previous studies by Dr. John Kinross at HWU (unpublished observations) found 3.5 mM MOPS buffer to stabilise pH, whilst displaying no toxic effects to the algae *Pseudokirchneriella subcapitata*. In this study, MOPS (3.5 mM) was found to have no toxic effect upon *L. variegatus* in 96 hour OECD medium exposures. Exposures of *L. variegatus* to NM-300K were conducted as described in section 3.2.5 at both pH 6.5 and 8.5 using the NM-300K LC_{50} dose (0.5 mg/L) to assess whether pH influenced NM-300K toxicity in relation to *L. variegatus.*

OECD medium with increased or reduced quantities of $CaCl_2$ $2H_2$, $MgSO_4$ $7H_2O$, $NaHCO_3$ and KCl stocks were prepared to give conductivities of 50, 100, 200, 450, 800 and 900 µS/cm in order to investigate the influence of medium ionic strength on NP toxicity to *L. variegatus*. OECD medium was aerated overnight before use, after which conductivity and pH (which varied according to conductivity) were measured. Using data generated from section 3.2.5, each medium was spiked with the LC_{50} recorded for NM-300K in standard OECD medium (0.5 mg/L). Exposures (96 hour) were conducted as described in section 3.2.5. Both conductivity and pH were measured after 96 hours, whilst controls for each medium were run in parallel to investigate whether changes in conductivity and pH caused toxicity despite the absence of nanoparticles.

3.2.9 Sub-lethal effects on L. variegatus behaviour.

Based on the results generated from section 3.2.5, *L. variegatus* were exposed to a sub-lethal dose of NM-300K (0.05, 0.1 and 0.2 mg/L) or NM-104 (0.5, 1 and 2 mg/L) as described previously before the behavioural responses; body reversal and helical swimming were assessed following 2, 6, 24, 48 and 72 hours of exposure. *L. varegatus* were also exposed to NM-300K (0.2 mg/L) in the presence of 5 mg/L SRHA OECD medium to investigate whether the presence of NOM influenced *L. variegatus* behaviour. Ten worms were individually exposed to the test substance or the control (OECD medium) after which, worms were transferred to a glass petri dish and allowed to acclimatise for approximately 30 seconds. Anterior and posterior ends of worms were then alternately touched gently with a wooden pick 5 times each to evoke behavioural responses. Time between touches, approximately 10 seconds, were allowed so as not to cause distress or promote erratic behaviour. Exposures were set up in a randomised and coded manner to eliminate the possibility of any subconscious bias when assessing behavioural responses.

3.2.10 Statistical analysis

Data was assessed for normality and homogeneity of variance using the Shapiro-Wilk test. Concentration-response curves (four-parameter logistic) were fitted in GraphPad Prism® 6. Response values were normalised and plotted against the logarithm of NM-300K concentration, from which LC_{50} values were generated. One-way ANOVA and Tukey test were used to detect the presence of statistical differences between mortality rates at different conductivities (IBM SPSS Statistics 22), while linear regression analysis was performed using GraphPad Prism® 6. Two-way ANOVAs using GLM were emplyed to detected significant differences in *L. variegatus* behaviour according to NP concentration/media type and time. Whenever significant, Tukey tests were applied to detect where differences were apparent. To specifically identify significant differences, individual one-way ANOVA and Tukey tests were performed for behavioural responses at each time point (IBM SPSS Statistics 22).

3.3 Results

3.3.1 L. variegatus NM-300K toxicity

In pilot studies, 5 concentrations of NM-300K were tested (0.01-100mg/L), with mortality and behavioural responses assessed 96 hours' post exposure. Concentrations of 0.01 and 0.1mg/L did not induce mortality to *L. variegatus*. An 80% mortality rate with no successful behaviour responses observed at a concentration of 1 mg/L NM-300K after 96 hours. At the two highest concentrations (10 and 100 mg/L) 100% mortality was recorded and hence, no behavioural responses were observed (table 3.1). These data were used to identify an appropriate range of NM-300K concentrations for the full toxicity testing (0-1.5 mg/L) for more in-depth toxicity testing, resulting in the concentration-response curve presented in figure 3.2. The lowest concentration to evoke *L. variegatus* mortality was 0.25 mg/L (6.66 %), whilst the highest concentration (1.5 mg/L) inflicted 100% mortality. An LC_{50} value of 512 µg/L was calculated. NM-300K DIS induced no *L. variegatus* mortalities at the highest test concentration, ruling out any toxic effects of the dispersant (data not shown).

Table 3.1. Pilot study for NM-300K toxicity towards *L. variegatus* following 96 hours exposure n=5.

NM-300K concentration (mg/L)	Mortality (%)	Behavioural response (%)
0	0	96.66
0.01	0	63.33
0.1	0	63.33
1	80	n/a
10	100	n/a
100	100	n/a

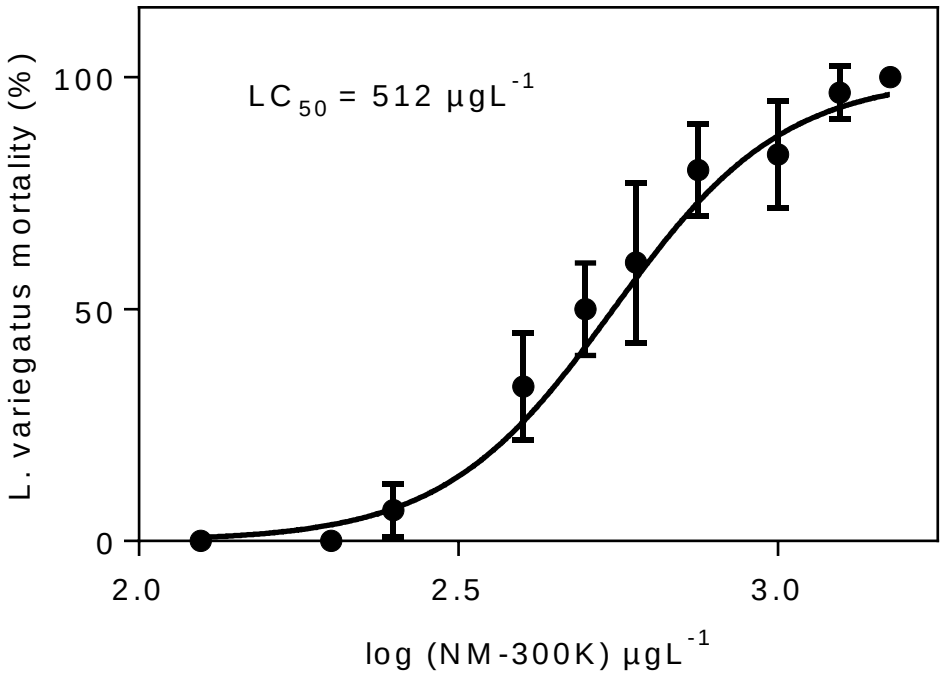

Figure 3.2. Concentration-response curve displaying mean *L. variegatus* mortality following 96-hour NM-300K exposure (0-1500 µg/L) (Data expressed as 100% of control (OECD medium) ±SD, n= 3)

3.3.2 L. variegatus NM-104 toxicity

The results of NM-104 pilot studies (table 3.2) indicated no toxicity towards *L. variegatus* at any of the concentrations tested, despite the inclusion of extremely high test concentrations (up to 1.5 g/L). NM-104 was found to quickly aggregate/agglomerate (assessed visually) and settle to the bottom of test vials at each concentration. As no toxicity was observed, no concentration-response relationship could be induced.

Table 3.2. Pilot study for NM-104 toxicity towards *L. variegatus* following 96 hours exposure (n = 1).

NM-104 concentration (mg/L)	Mortality (%)
0	0
7.8	0
15.6	0
31.25	0
62.5	0
125	0
250	0
500	0
1500	0

3.3.3 L. variegatus $AgNO_3$ toxicity.

Pilot studies using a concentration range based on that used for NM-300K (section 3.3.1) displayed the highly toxic nature of $AgNO_3$. At the lowest test concentration (0.05mg/L), 70% mortality was recorded and 100% mortality was recorded at concentrations of 0.25 mg/L and above (table 3.3). Most *L. variegatus* mortality occurred within the first few of hours of exposure.

Table 3.3 Pilot study for $AgNO_3$ toxicity towards *L. variegatus* following 96-hour exposure, n=1.

$AgNO_3$ Concentration (mg/L)	Mortality (%)
0	0
0.05	70
0.125	80
0.25	100
0.5	100
0.75	100
1	100

Based on the results generated from the pilot study (table 3.3), a full toxicity test was conducted for $AgNO_3$ using an amended concentration range of (0-175 µg/L). From the resultant dose-response curve (figure 3.3), an LC_{50} value of 35.30 µg/L was identified. A concentration dependent increase in mortality was observed, with 100% mortality observed at a concentratioin of 175 µg/L.

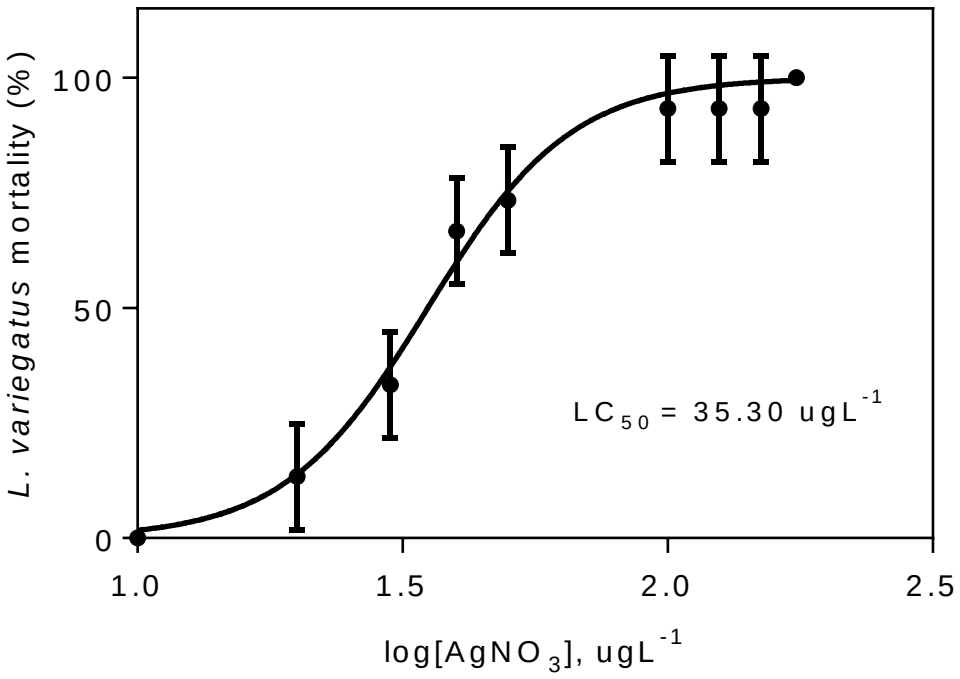

Figure 3.3 Concentration-response curve displaying mean *L. variegatus* mortality following 96 hour $AgNO_3$ exposure (0-175 µg/L). (Data expressed as 100% of control (OECD medium) ±SD, n= 3)

3.3.4 Influence of NOM on NM-300K toxicity

Pilot NOM experiments demonstrated a SRHA concentration-dependent influence upon NM-300K toxicity (figure 3.4). An increase in SRHA reduced the toxicity of NM-300K. For example, a concentration of 1.5 mg/L NM-300K was previously found to induce 100% *L. variegatus* mortality in standard OECD medium, however in the presence of 5 and 50 mg/L SRHA, mortality was reduced to 80 and 40%, respectively (figure 3.4).

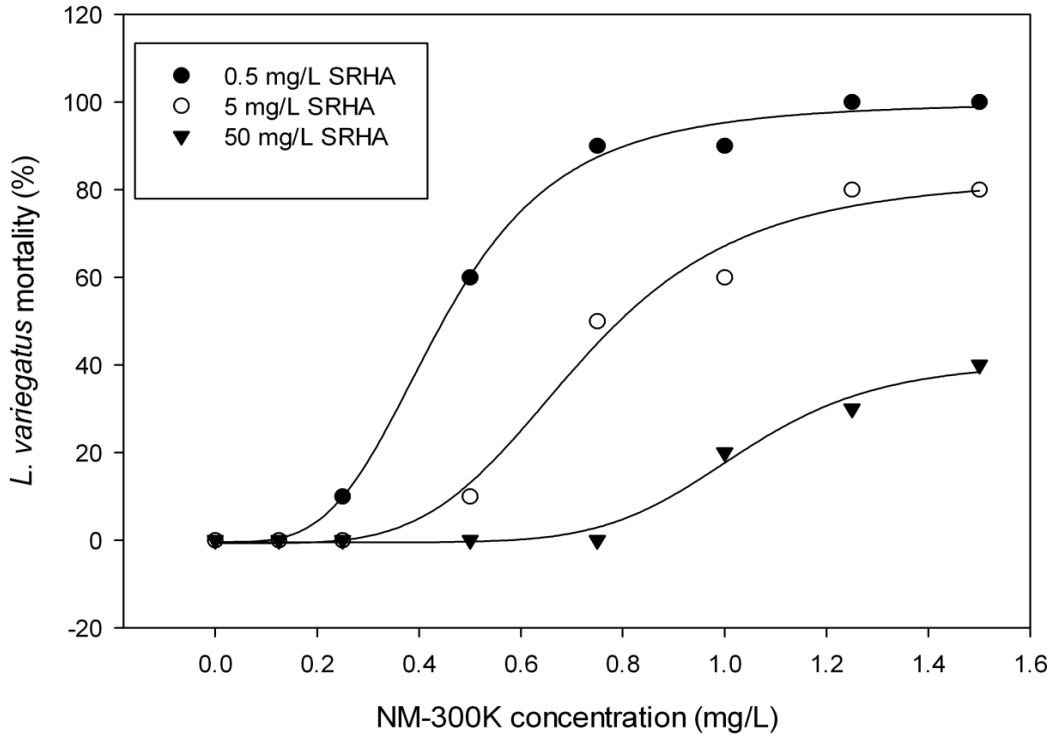

Figure 3.4 Concentration-response relationships of *L. variegatus* exposed to NM-300K (0-1.5 mg/L) in OECD media containing 0.5, 5, and 50 mg/L SRHA (± SD, n=1).

A full toxicity test was then performed using 5 mg/L SRHA OECD medium for direct comparison with toxicity data for NM-300K suspended in OECD medium containing no SRHA (figure 3.5). Although 1.5 mg/L NM-300K was found to evoke 100% mortality in

worms exposed in standard OECD medium after 96 hours, 86.66% mortality was observed in 5 mg/L SRHA OECD medium. However, this finding was not significant ($p>0.05$). Similarly, 250 µg/L NM-300K was the lowest concentration found to induce mortality (6.66%) in standard OECD medium, whilst no mortality was observed in 5 mg/L SRHA OECD medium until 500 µg/L (23.33%). Beyond 500 µg/L NM-300K results for OECD and OECD with SRHA were more comparable and consequently, no statistical difference was detected between the LC_{50} values generated (512 µg/L and 569.7 µg/L, respectively, using t-test comparing best fit variables – GraphPad Prism 6 ®) ($p>0.05$), despite the more pronounced mitigating effects of SRHA at lower test concentrations.

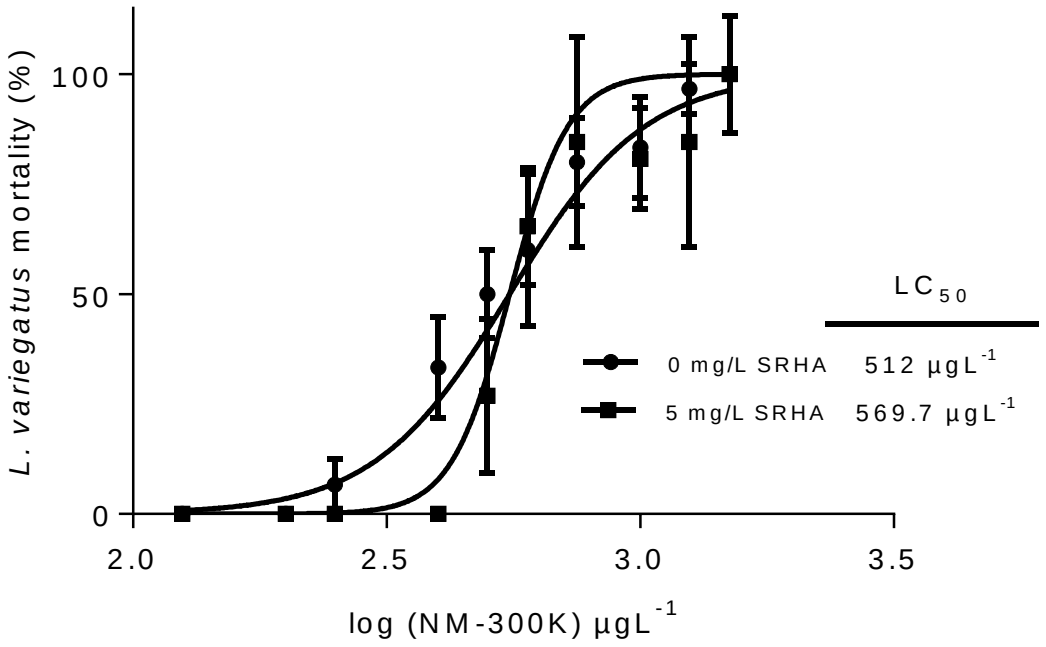

Figure 3.5 Concentration-response relationships of *L. variegatus* exposed to NM-300K (0-1500 µg/L) in OECD medium containing 0 and 5 mg/L SRHA. (Data expressed as 100% of control (OECD medium) ±SD, n= 3)

3.3.5 pH and ionic strength

Increasing media conductivity was found to coincide with increasing pH values (table 3.5) with 50 and 950 µS/cm OECD medium recording the lowest (6.4) and highest (8.32) pH values respectively (pre-exposure). Paired sample t-tests detected significant changes ($p<0.05$) in conductivity, pH, or both following 96 hours across all media, apart from 50 µS/cm medium. One-way ANOVA results detected a significant difference in *L. variegatus* mortality when exposed in differenct ionic strength OECD medium ($p<0.005$). Following a 96-hour exposure to 0.5 mg/L NM-300K (LC_{50} value in standard OECD medium), only exposures in 50 and 950 µS/cm media recorded significantly different changes in *L. variegatus* mortality (figure 3.6) compared to standard OECD medium (using Dunnett tests, $p<0.05$). More specifically, mortality increased to 70% in 50 µS/cm medium, whilst 950 µS/cm mortality was only 23.33%, which was significantly lower ($p<0.05$) than mortalities in all other media strengths.

Table 3.4 Mean conductivity and pH measurements for different strength OECD media before and after 96-hour NM-300K, *L. variegatus* exposures (± SD, n=3). Asterisks symbolise significant differences following paired-sample t-test ($p<0.05$) between pre- and post exposure measurements.

Conductivity (µS/cm)	Conductivity (µS/cm)		pH	
	Pre-exposure	Post-exposure	Pre-exposure	Post-exposure
50	51.71 (±0.40)	60.51 (±11.01)	6.40 (± 0.006)	6.51 (±0.39)
100	100.76 (±0.28)	109.21 (±1.41) *	6.55 (±0.003)	6.42 (±0.14)
200	199.65 (± 1.10)	214.95 (±0.61) *	7.00 (±0.008)	7.00 (±0.04)
450	445.83 (±3.12)	468.33 (±56.76)	7.62 (±0.06)	7.48 (±0.12) *
700	684.83 (±1.94)	695.66 (±3.44) *	7.76 (±0.01)	7.70 (±0.05) *
800	802 (±1.26)	799.33 (±11.11)	7.87 (±0.12)	7.76 (±0.02) *
950	937.33 (±5.1)	953.16 (±5.19) *	8.32 (±0.01)	7.69 (±0.11) *

Although 950 µS/cm medium recorded a pH significantly higher than all other media before exposure (8.32), it was found to significantly decrease over 96 hours, becoming similar to that of 700 and 800 µS/cm OECD medium *(*p>0.05). Despite this, *L. variegatus* mortality was still significantly lower in 950 µS/cm medium, strongly indicating that increased survival was a consequence of ionic strength alone.

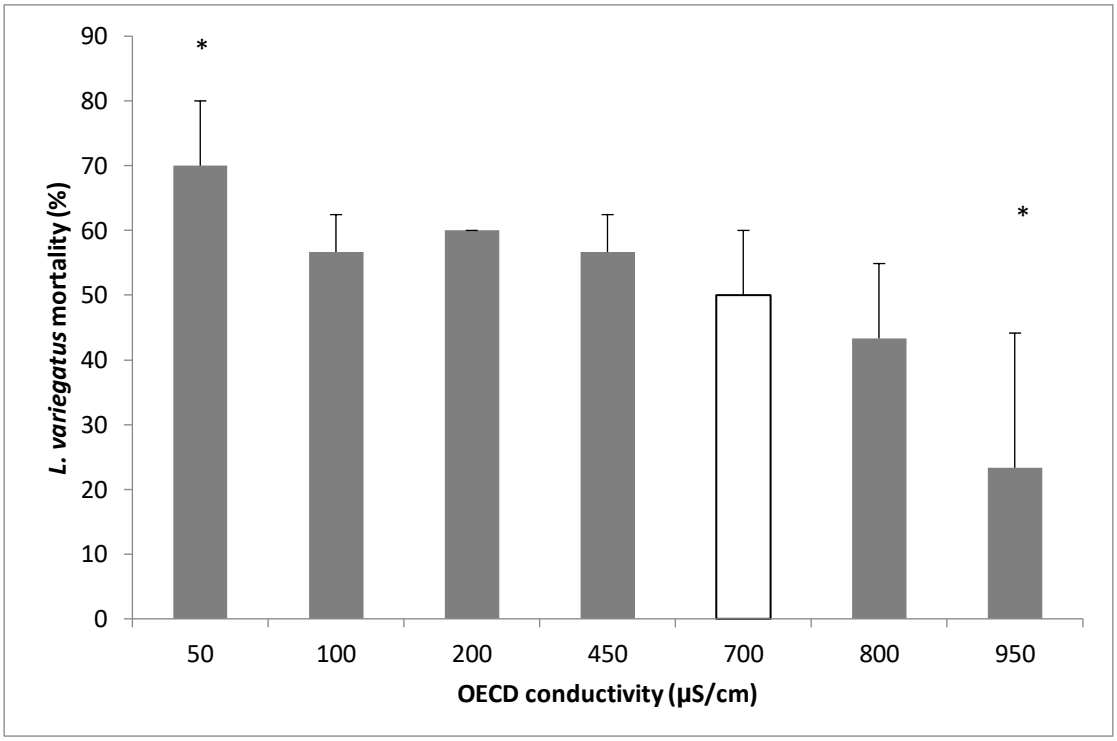

Figure 3.6 Mean *L. variegatus* mortality following 96-hour exposure to 0.5 mg/L NM-300K in OECD media of differing strength (±SD). Asterisks denote significant (p<0.05) differences in mortality compared to standard OECD medium (700 µS/cm). Data expressed as % mortality (% of control), n= 3

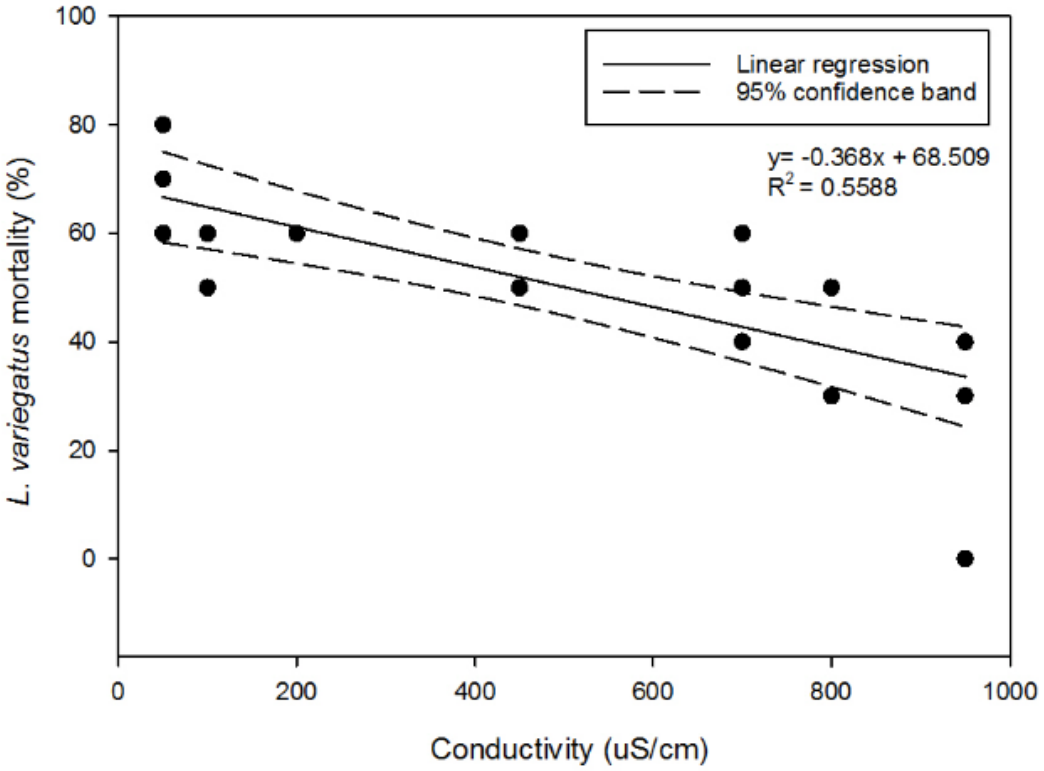

Figure 3.7 Linear regression of OECD media conductivity and *L. variegatus* mortality following 96-hour exposure to 0.5 mg/L NM-300K, n=3.

Linear regression analysis displayed a correlation between OECD conductivity and *L. variegatus* mortality following exposure to 0.5 mg/L NM-300K. An R^2 value of 0.5588 indicated that 55.88% of the variation in *L. variegatus* mortality can be explained by OECD media conductivity.

3.3.6 NM300K *L. variegatus behaviour*

Differences between the body reversal behaviours of worms exposed to NM-300K at various concentrations and time points are presented in figure 3.8A and B. Two-wy ANOVA analysis demonstrated that the combination of media and time was significant in determining both *L. variegatus* body reversal and helical swimming ($p<0.0001$). Tukey tests detected significant differences ($p<0.05$) in body reversal and helical swimming for *L. variegatus* in all medium tested, with the exception of 0.05 and 0.1 mg/L, where no differences were detected ($p>0.05$).

Individual one-way ANOVA and Tukey tests were performed for each time-point to detect whether significant differences existed between behavioural responses for *L. variegatus* in each medium. Worms exposed to both 0.1 and 0.2 mg/L NM-300K recorded significantly worse body reversal responses than controls at each time point, whilst those exposed to 0.05 mg/L performed significantly worse after 2, 24 and 72 hours of exposure (figure 3.8A). Increasing NM-300K concentration also generally lead to a decline in *L. variegatus* helical swimming responses, however results proved to be more variable (figure 3.8B). As with body reversal, 0.2 mg/L results were significantly poorer ($p<0.05$) than all other media after 24 hours. The only time-point to record no significant differences was after 48 hours.

Individual one-way ANOVA and Tukey tests were performed to determine whether significant differences in behaviour existed between time points for *L. variegatus* exposed to each medium. The only evident pattern existed for worms exposed to 0.2 mg/L NM-300K. Significant differences ($p<0.05$) in body reversal values were detected between 24 hours (6.66% success) and every other timepoint, apart from 72 hours of exposure (33.33%). Similarly, significant differences ($p<0.05$) in helical swimming were detected between 24 hours (4.4 %success) and every other time-point with the exception of 6 hours (44.44%). These results indicate a potential improvement in *L. variegatus* behaviour (48 and 72 hours) following a dramatic decline after 24 hours exposure to 0.2 mg/L NM-300K.

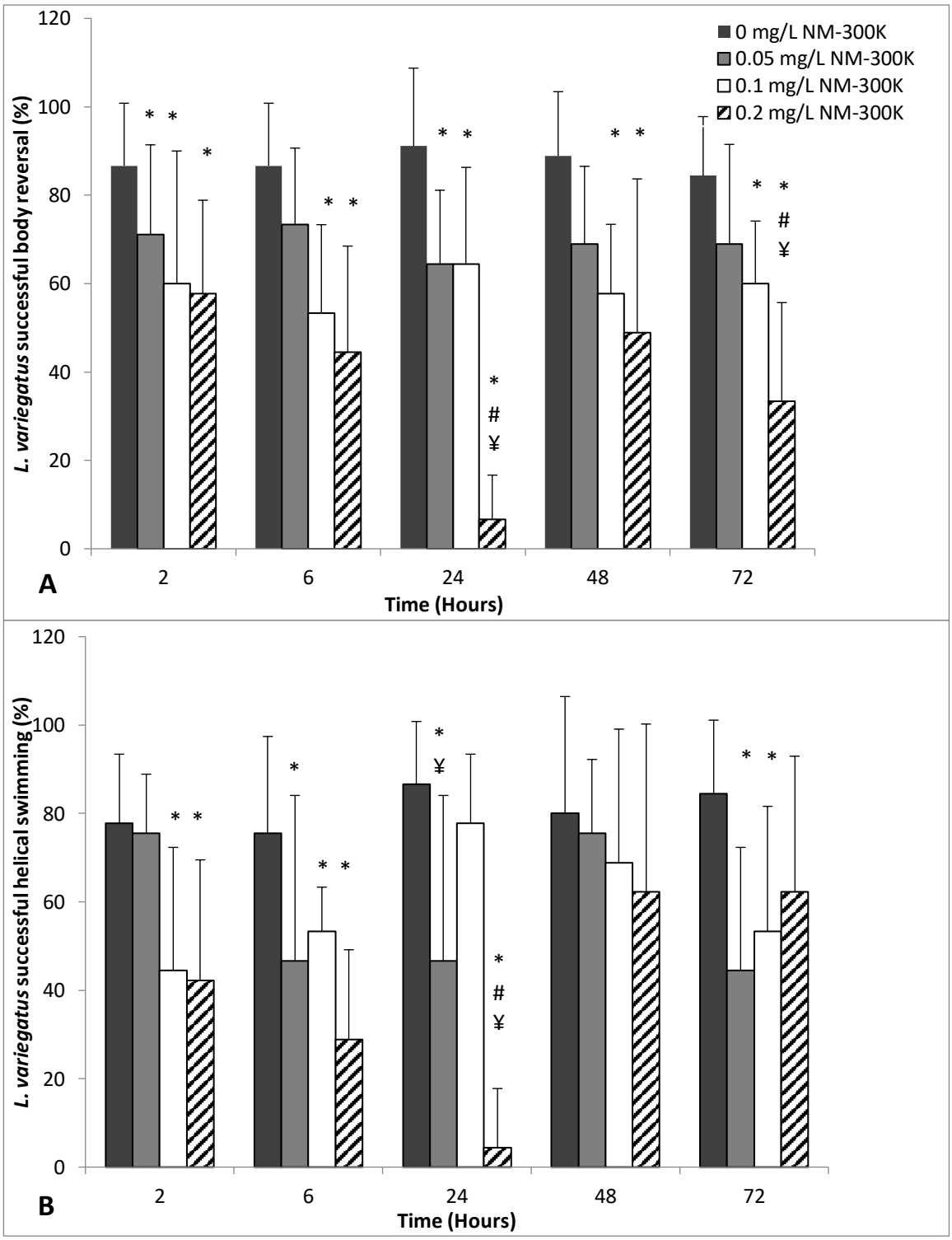

Figure 3.8 Mean successful body reversal (A) and helical swimming (B) of *L. variegatus* exposed to; 0, 0.05, 0.1 and 0.2 mg/L NM300-K after; 2, 6, 24, 48 and 72 hours of exposure (+ SD, n=3). Symbols at each time point denote significant difference ($p<0.05$) to control (*), 0.05 mg/L (#) and 0.1 mg/L (¥).

3.3.7 NM-300K SRHA OECD L. variegatus behaviour

The inclusion of 5 mg/L SRHA in OECD medium had a profound effect upon *L. variegatus* behaviour during NM-300K exposures. Significant differences ($p<0.0001$) were detected (via two-way ANOVA) between body reversal and helical swimming responses of *L. variegatus* when in different media: control (OECD medium, no NM-300K), 0.2 mg/L NM-300K in OECD medium and 0.2 mg/L NM-300K in OECD medium containing 5 mg/L SRHA. Tukey tests detected these differences to exist between each of the aforementioned media ($p<0.05$). One-way ANOVA and Tukey analysis at each time point detected significantly poorer responses in SRHA OECD medium containing NM-300K (compared to control) after only 2 and 6 hours (body reversal) and 2 hours (helical swimming – figure 3.9 A and B, respectively). Furthermore, when comparing the body reversal responses of 0.2 mg/L exposures with and without 5 mg/L SRHA, those without were found to be significantly worse ($p<0.05$) after 6, 24, 48 and 72 hours. Similar findings were found for helical swimming responses after both 6 and 24 hours.

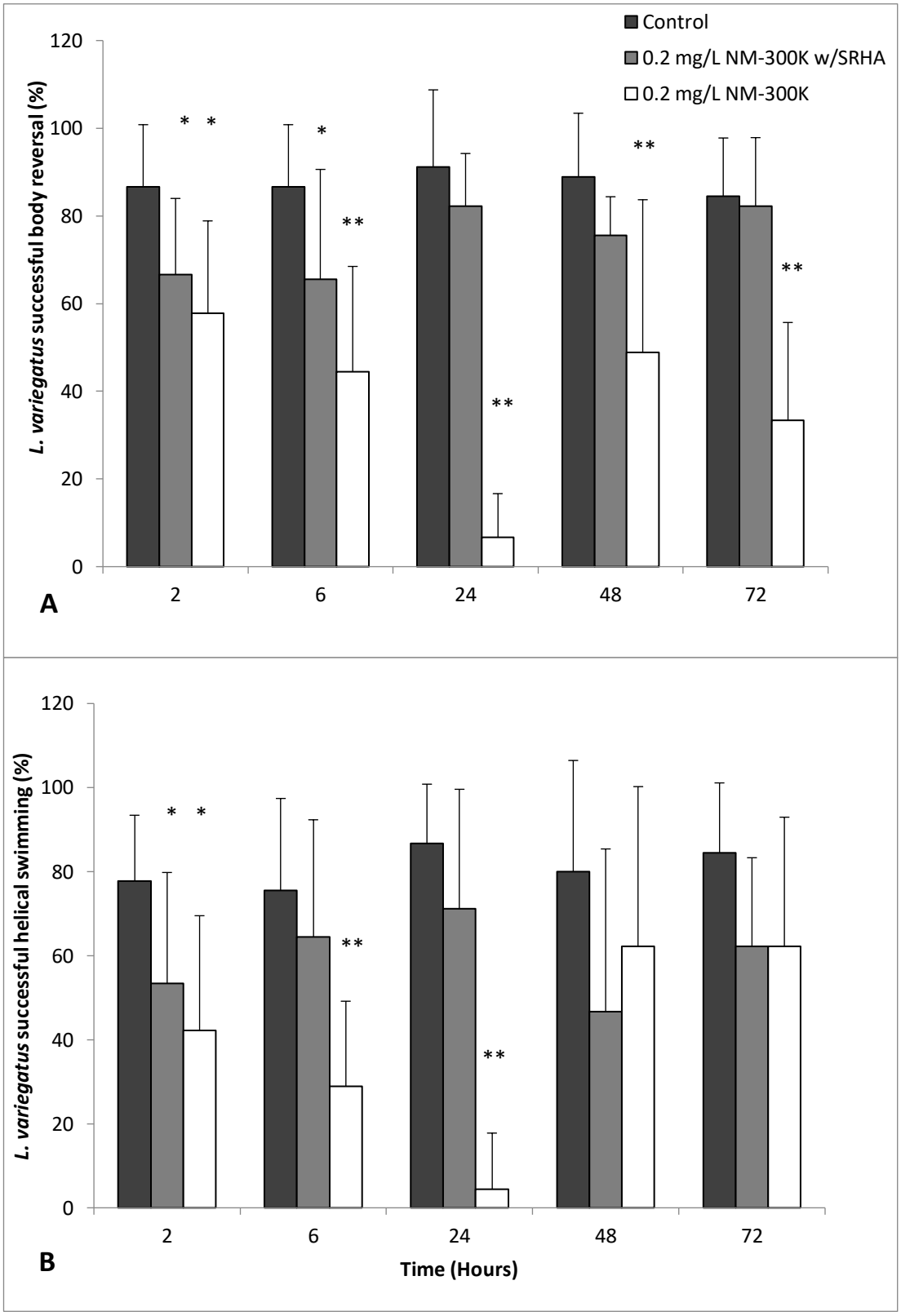

Figure 3.9 Mean successful body reversal (A) and helical swimming (B) of *L. variegatus* exposed to 0 and 0.2 mg/L NM-300K in OECD medium with and without 5 mg/L SRHA after 2, 6, 24, 48 and 72 hours of exposure (+ SD, n=3). * denotes significant difference from control, ** denotes significant differences from both control and 0.2 mg/L NM-300K.

3.3.8 NM-104 behaviour

Two-way ANOVA analysis found medium (control or 0.2 mg/L NM-104) and time (2-72) to have no influence on body reversal or helical swimming behaviours ($p<0.0001$). Despite 2 mg/L NM-104 exposure, successful body reversal remained over 80% throughout the 72-hour period (figure 3.10A) and over 65% for helical swimming (figure 3.10B).

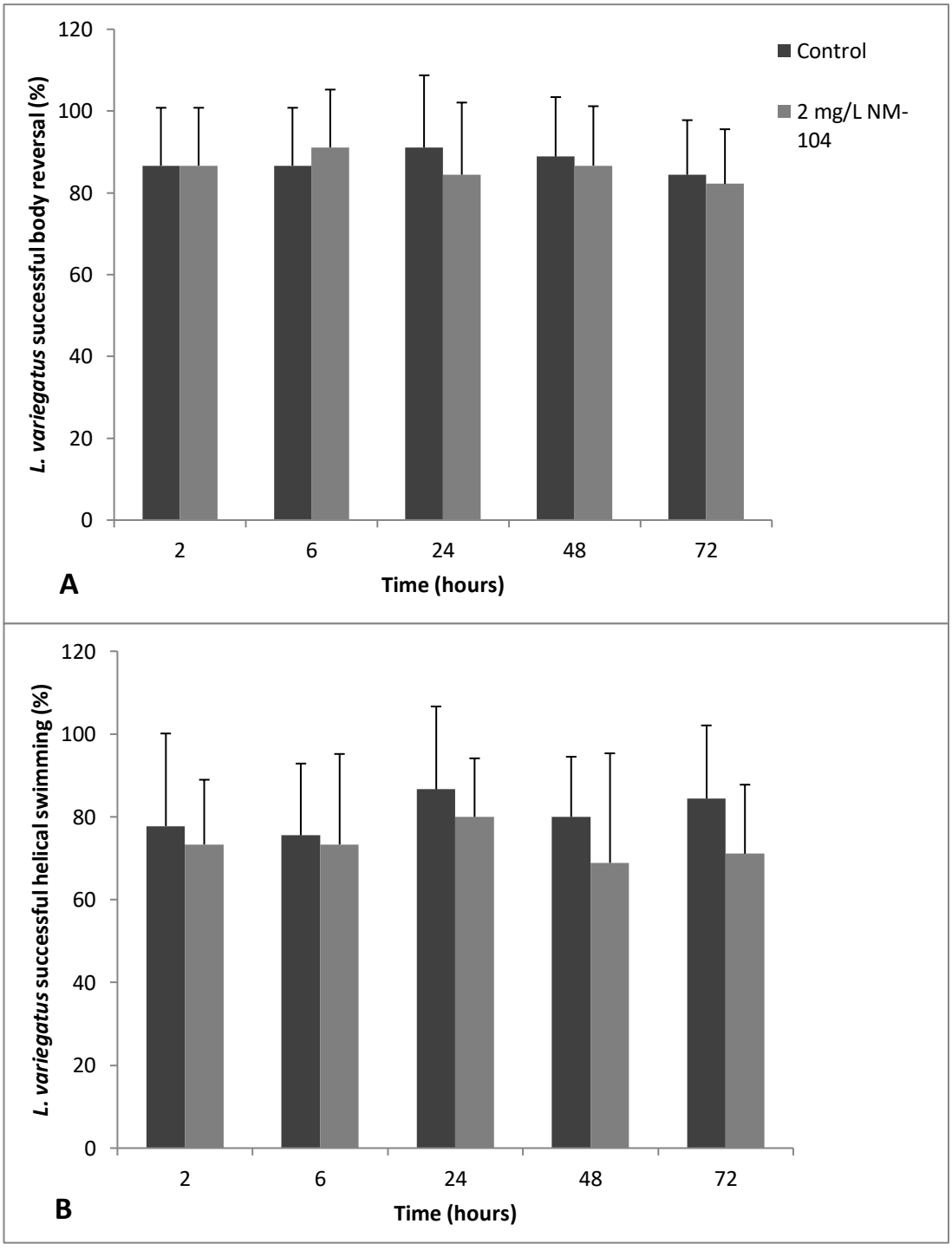

Figure 3.10 Mean successful body reversal (A) and helical swimming (B) of *L. variegatus* exposed to; 0 and 2 mg/L NM-104 after; 2, 6, 24, 48 and 72 hours (+SD, n=3).

3.4 Discussion

3.4.1 $AgNO_3$ and NM-300K toxicity

One of the primary concerns in regards to the environmental release of AgNPs is whether potential toxicity is related directly to particle mediated effects, or are the product of dissolved silver ions released from the particle over time. In the case of the latter, the risk assessment of silver nanoparticles could be categorised using existing regulations, if it was considered that these NPs would fully dissolve. If toxicity is primarily mediated by particle effects, Addionally, novel modes of toxicity may emerge and call for nano-specific risk assessments to be derived. To gain a holistic view of NM-300K toxicity in relation to *L. variegatus,* the investigation of dissolved silver (in the form of $AgNO_3$) toxicity was also considered as part of this research.

A concentration-dependent increase in *L. variegatus* mortality was observed following exposure to $AgNO_3$ and NM-300K after 96 hour exposures. However, $AgNO_3$ was found to be almost 15 times more toxic as indicated by the LC_{50} values; 35.3 µg/L and 512 µg/L, respectively. As reported in chapter 2, the dissolution of Ag from NM-300K after 96 hours in OECD medium was found to be 3.14%. Based on these findings, a NM-300K concentration of 512 µg/L (the LC_{50} value) would lead to a dissolved fraction with a concentration of 16.07 µg/L Ag^+. Whilst this dissolved fraction may be accountable for some of the toxicity observed in NM-300K exposures, it cannot be considered the sole cause (as $AgNO_3$ was found to have an LC_{50} value of 34.05 µg/L). These results suggest that NM-300K offers a mode of toxicity novel to that of Ag^+, most likely in relation to particle size and the associated physicochemical properties. Toxicity tests for NM-300K DIS confirmed no mortality of *L. variegatus* exposed to the dispersant alone for 96 hours (at the equivalent amount present in 1.5 mg/L NM-300K – a concentration known to lead to 100% mortality). These findings further confirm the notion that the mortalities incurred were a product of exposures to nanoscale silver, either directly or in combination with dissolved Ag^+.

Although no published studies have made comparisons between the toxicity of dissolved and nanoscale silver for *L. variegatus* in aquatic exposures, comparable findings have been reported in alternate freshwater species. For example, Navarro *et al* (2008) identified that the toxicity of $AgNO_3$ towards the freshwater algae,

Chlamydomonas reinhardtii was 18 times higher than AgNP (EC_{50} values of 188nM and 3300nM, respectively, after a 1 hour exposure). In addition, they also reported that Ag^+ dissolution (measured at 1%) could not fully account for the toxicity exhibited by the AgNP. Similarly, Griffitt *et al* (2008) concluded that whilst soluble Ag was found to be more toxic than AgNP towards various freshwater species (zebrafish, daphnia and algal species), the mortality evoked by AgNP could not be attributed solely to dissolution, the rate of which was found to be relatively low (0.07% of mass). Conversely, whilst Zhao and Wang (2011) found $AgNO_3$ to be significantly more toxic than AgNP towards *D. magna* ($AgNO_3$ 48 hour LC_{50} of 2.51 µg/L, no mortalities in up to 500 µg/L AgNP), they hypothesised that any potential AgNP toxicity would occur as a result of Ag^+ dissolution alone. Additionally, after the removal of Ag^+ from AgNPs, Kim *et al* (2011) found no acute toxicity towards *D. magna*, indicating that previously witnessed acute effects could be attributed exclusively to Ag^+.

Other comparisons between the toxicity of $AgNO_3$ and AgNPs towards freshwater species have generated alternate conclusions, whereby AgNP toxicity has shown to be comparable to, or greater than $AgNO_3$. Whilst Farkas *et al* (2011) found $AgNO_3$ and AgNP to display similar dose-response relationships in rainbow trout (*Oncorhynchus mykiss*) exposures, greater toxicity was exhibited by AgNPs at higher concentrations, despite low levels of Ag^+ dissolution. This was accredited to the fact that AgNPs can be taken up directly by cells (Hsin *et al,* 2008), release Ag^+ and promote cytotoxicity to a greater extent that $AgNO_3$, which, at higher concentrations may be limited to uptake via ion channels (Bury and Wood, 1999). Farkas *et al* (2011) also highlight the possibility of AgNPs adhering to the surface of cells, enabling the release of Ag^+ within close proximity without the limitations of diffusion. This is important to consider in relation to the toxicity of NM-300K towards *L.* variegatus.

Differentiating between ion and particle effects towards organisms is an area of great interest within nano-toxicology. One of the primary modes through which AgNPs are considered to stimulate toxic effects alternate to those of Ag^+ is through the penetration of cells, causing, the generation of reactive oxygen species (ROS), their associated effects and their interaction with, and inhibition of, specific enzymes and proteins (van Aerle *et al,* 2013).

While the direct toxicity of NM-300K towards *L. variegatus* is important, the indirect effects of potential NM-300K release into freshwater environments must also be considered. The well noted anti-bacterial properties of Ag could have a profound effect upon sediment bacteria (and other organisms) that act as a food source for *L. variegatus.* The decline in such sediment biota could have major implications, not only towards *L. variegatus* populations but for the ecological functioning of freshwater environments.

3.4.2 Influence of SRHA on NM-300K toxicity

Although pilot SRHA toxicity tests revealed a clear relationship between SRHA concentration and NM-300K toxicity towards *L. variegatus*, no significant differences were detected between the LC_{50} value of NM-300K in 0 and 5 mg/L SRHA OECD media during full toxicity testing. This finding goes against the consensus within the literature whereby NOM is considered to mitigate NP toxicity towards freshwater species. For example, Van Hoecke *et al* (2010) found EC_{20} values (4.7-395.8 mg/L) of *Pseudomonas fluorescens* exposed to cerium oxide NPs to increase linearly with an increase in NOM concentration (0-7.5 mg C/L) within OECD algal medium; whilst Gao *et al* (2012) found a linear decrease in AgNP toxicity towards *D. magna* following an increase in SRHA concentration (up to 20 mg TOC/L).

Estimating concentrations of humic acid within freshwaters is challenging given their transient nature e.g. changing per geographical location and climate (Visser, 1984). Humic substances are known to account for most (50-80%) of the dissolved organic matter in freshwater ecosystems (Steinberg *et al,* 2006). Freshwater ecosystems rich in humic substances can reach dissolved organic concentrations of approximately 160 mg/L (albiet with median values of 5.7 mg/L) (Suhett *et al,* 2004), whilst HA in oligotrophic water bodies can range from 0.5 mg/L to 80 mg/L (Ferguson, 2011). A concentration of 5 mg/L HA was selected within this investigation as it represents a somewhat conservative concentration. However, whilst a significantly similar LC_{50} value was recorded for NM-300K in 5 mg/L SRHA medium and medium containing no SRHA, it still must be recognised that no *L. variegatus* mortalities were observed under a concentration of 500 µg/L, NM-300K whilst the highest test concentration of NM-300K (1.5 mg/L) was unable to evoke 100% mortality (as observed in OECD medium containing

no SRHA). Results of the pilot study indicate that a decrease in NM-300K toxicity was most likely evident with an increase in SRHA concentration, similar to the findings of Gao *et al* (2012) and Van Hoecke *et al* (2010). It is therefore possible that the use of a greater SRHA concentration would have resulted in significantly less toxicity in regards to the LC_{50} value generated.

Characterisation of NM-300K suspended in 5 mg/L SRHA OECD medium via TEM imaging revealed no apparent SRHA coating of dispersed particles, whilst the zeta potential was found to be significantly less negative than particles suspended in OECD medium containing no SRHA. These findings indicate that steric repulsion of NM-300K particles was not enhanced in the presence of 5 mg/L SRHA. Unlike Liu and Hurt (2010), who observed NOM to adsorb to AgNP particles and consequently limit their release of ionic silver, dissolution of NM-300K was not found to be significantly different in OECD medium containing 0 and 5 mg/L SRHA. Despite these findings, TEM images confirmed there to be large filamentous structures within the medium (most likely decaying vegetation), to which NM-300K particles had adhered to (as confirmed via EDS). Particles within these agglomerates/aggregates will undoubtedly become coated with SRHA, altering their surface charge (it is possible that due to their size, SRHA aggregates may have settled during DLS measurements and gone undetected). Furthermore, the absorption of particles to SRHA structures may limit their availability, and hence, toxicity, towards *L. variegatus,* particularly at higher concentrations. Humic acid also possesses chelating properties towards metals and metalic ions (Dubas and Pimpan, 2008) and could thus make Ag^+ ions released from NM-300K less bioavailable, further limiting toxicity.

3.4.3 Influence of media composition on NM-300K toxicity

OECD media of low pH and ionic strength were found to induce greater mortality to *L. variegatus*, whilst an increase in ionic strength and pH were found to decrease it. Although pH studies evoked changes in *L. variegatus* mortality, the introduction of 1M NaOH (used to raise pH) was also found to significantly raise the ionic strength of OECD medium. Standard OECD typically records a conductivity of ~700 µS/cm, however pH-altered OECD medium recorded conductivities of ~1200 µS/cm. Changes in toxicity

could therefore not be attributed to a change in pH alone. Consequently, experiments were conducted investigating ionic strength in conjunction with pH. Lower toxicity of AgNPs in high ionic strength medium is considered to be a result of altered aggregation states (Fabrega *et al,* 2011), a theory that corresponds with the findings of this research.

Within colloid science, divalent ions such as Ca^{2+} are well known to facilitate particle agglomeration/aggregation and have demonstrated such in other metal oxide NPs (Zhang *et al,* 2009). Furthermore, Cl^- has demonstrated an ability to encourage bridges between AgNPs, forming $AgCl^-$ precipitates, mitigating the toxic effects of AgNPs (Bradford *et al,* 2009). One of the major components of OECD medium, $CaCl_2$, acts as a source of both Ca^{2+} and Cl^- whilst the remaining components ($MgSO_4$, $NaHCO_3$ and KCl) supply additional cations (Mg^{2+}, K^+ and Na^+, respectively). When the concentration of such counterions was increased in OECD medium (950 µS/cm), the zeta potential was found to have more neutral values, whilst more dilute OECD medium (50 µS/cm) recorded significantly more negative ZP values (-12.7 mV and -21.6 mV, respectively, after 96 hours).

In cases where ionic strength is increased and zeta potential becomes more neutral (due to the shielding of particles - in accordance with the DLVO theory), the diffuse layer thickness of particles is reduced, allowing NPs to come in close enough proximity to aggregate. Reduced toxicity of NM-300K towards *L. variegatus* when exposed in 950 µS/cm medium (figure 3.6) is inextricably linked to aggregation, whereby the surface area of aggregates/agglomerates is less than the combined surface area of primary particles, limiting the toxic potential. Conversely, in 50 µS/cm OECD medium, the more negative surface charge of particles (a result of the low counterion concentration) led to a greater retention of electrostatic repulsion between particles, improving their stability and limiting their aggregation/agglomeration. In a more stable, dispersed state, NM-300K particles remained within the nano-scale and could exhibit any potentially size-dependent toxic effects towards *L. variegatus.*

Greater aggregation/agglomeration of NPs is commonly regarded to mitigate NP toxic effects, and has been demonstrated in bacteria (Lok *et al,* 2007); Bradford *et al,* 2009), and zebrafish, where Griffit *et al* (2009) found the majority of metallic NPs (including Ag) to be removed from the water column shortly after application due to aggregation and settlement. Although OECD medium represents an environmentally relevant medium,

both pH and ionic strength are transient throughout the environment. Indeed *L. variegatus* are known to inhabit a wide range of freshwater ecosystems and therefore it is important to investigate how environmental conditions may affect NP fate, thus exposure and biovavailability. Consequently, understanding the relationship between media composition and NM-300K toxicity was important as part of this research.

3.4.4 NM-104 toxicity

Titanium dioxide nanoparticles (NM-104) exhibited no toxicity towards *L. variegatus* in 96-hour aquatic exposures. *L. variegatus* could survive concentrations of NM-104 up to 1.5 g/L, a concentration highly unlikely to be experienced within the environment. These findings were in keeping with the literature, where relatively high concentrations of TiO_2NPs have also been found to have little effect upon the survival, growth, reproduction or function of several freshwater species, including; rainbow trout (Federici *et al,* 2007; Ramsden *et al,* 2009; Scown *et al,* 2009) zebrafish (Chen *et al,* 2011), and *D. magna* (Heinlaan *et al,* 2008).

The crystalline form of TiO_2 is thought to play a key role in their toxicity. Rutile TiO_2 (such as NM-104) is considered to be inert, whilst anatase is generally regarded to be more toxic owing to its greater photocatalytic activity (Liu *et al,* 2012). By absorbing light below 385 nm, anatase TiO_2NPs form an electron hole pair (Brock *et al,* 2000), which, after interacting with water or oxygen will generate ROS (Konaka *et al,* 1999). The difference in crystalline structure has been demonstrated to have ecotoxicological effects, with Bang *et al* (2011), finding greater toxicity of anatase TiO_2NPs in comparison to rutile using *D. magna,* although toxicity may also have been related to size. Similarly, Clément *et al* (2012) found anatase TiO_2NP to be significantly more toxic than rutile TIO_2NP toward *D. magna, Phaeodactylum tricornutum* and *Brachionus plicatilis*. The lower toxicity exhibited by rutile TiO_2NP was accredited to its tendency to form large aggregates and greater surface energy.

Characterisation data presented in chapter 2 revealed NM-104 (which is rutile) to aggregate rapidly upon introduction to both Milli-Q water and OECD medium. The observed sedimentation of NM-104 to the bottom of test vials further demonstrated their strong affinity for aggregation – suggesting that their transport within the

environment would be limited (making testing in the bethic system highly relevant – as investigated in chapter 5). Whilst aggregation mitigates the potentially toxic effects of NPs through the reduction of their reactivity and cellular interactions, the possible adhesion of NP aggregates to the *L. variegatus* exoskeleton offers an alternate risk. Filter feeding species such as *D. magna* are particularly susceptible to TiO_2NP adhesion, causing physical effects, a loss of mobility (Baun *et al,* 2008) and an inhibition of moulting (Dabrunz *et al,* 2011), whilst displaying negligible cellular, biochemical or macromolecular effects. Despite not possessing filtration apparatus (highly prone to aggregate adhesion), and being less active within the water column than *D. magna*, the NP coating of *L. variegatus* could adversely impact their mobility and thus their ability to escape predation.

3.4.5 L. variegatus behaviour

Although no mortality was observed in *L. variegatus* in NM-300K concentrations up to 0.2 mg/L, behavioural responses intended for the escape of predation were found to worsen at lower concentrations. Conversely, no detrimental effects upon behaviour were exhibited by *L. variegatus* exposed to 2mg/L NM-104. To date, the use of *L. variegatus* behavioural responses for the bioassessment of potential contaminants have largely been restricted to sediment studies in the form of burrowing (Dermott and Munawar, 1992), sediment avoidance (West and Ankley, 1998), and feeding behaviour (Jonker *et al,* 2009), however some instances of locomotive responses have been investigated in relation to lead avoidance (Gerhardt, 2007) and the toxicity of copper (O'Gara *et al,* 2004).

Similarly to the findings of O'Gara *et al* (2004), a clear concentration dependent decline in *L. variegatus* body reversal was evident following NM-300K exposure, however, helical swimming responses were shown to be considerably more variable. Of the two behavioural responses, O'Gara *et al,* (2004) found helical swimming to be the more sensitive, citing the greater complexity of movements involved as a possible explanation. Although in some instances, helical swimming was found to be more sensitive to NM-300K than body reversal in this study, the aforementioned variability meant this was difficult to quantify as part of this research. A decline and recovery

relationship was evident for the behavioural responses of *L. variegatus* exposed to 0.2 mg/L NM-300K across 72 hours, suggesting an ability to mitigate the toxic effects potentially incurred over time. These results highlight the importance of testing at multiple time-points. O'Gara *et al* (2004) also demonstrated the ability of *L. variegatus* to recover behavioural responses, which were found to improve following their transfer from copper exposures to uncontaminated water.

As outlined earlier, both body reversal and helical swimming are mediated by activation of motor neurons by the medial and lateral giant fibres (MGF and LGF). O'Gara *et al* (2004) demonstrated the ability of copper to significantly reduce the conduction velocity of both *L. variegatus* giant fibres over time. The findings of such research suggest that a loss in behavioural function is also likely to be associated with a lowering of giant fibre conduction velocity because of neurological damage. AgNPs are known to have an inhibitory effect upon voltage-gated sodium currents (Liu *et al,* 2009) which determine many neuronal properties, notably the generation of action potential and its propagation to synapse terminals (Yang *et al,* 2010). AgNP mediated inhibition of voltage-gated potassium currents, responsible for modifying neuronal excitability and activity have also been recorded (Liu *et al,* 2010). The potential inhibition of sodium and potassium currents by NM-300K may have caused a slowing of *L. variegatus* giant fibre conduction velocity and ultimately be responsible for the poor behavioural responses recorded, however, this would have to be investigated further.

The inclusion of SRHA had previously shown no significant effect upon the toxicity of NM-300K towards *L. variegatus* (in relation to LC_{50} value generated after 96 hours' exposure), however, behavioural responses were significantly better when exposed to 0.2 mg/L NM-300K in 5mg/L SRHA OECD medium as opposed to in OECD medium with no SRHA. These results suggest that whilst 5 mg/L SRHA was unable to significantly offset NM-300K toxicity in relation to *L. variegatus* mortality, it had a profound effect upon *L. variegatus* behaviour at a sub-lethal NM-300K concentration. Using sub-lethal concentrations of CuO NPs (50 and 100 mg/L), Pradhan *et al* (2015) found the feeding behaviour of the freshwater invertebrate *Allogamus ligonifer* to be improved in medium containing HA (100 mg/L) as opposed to medium containing CuO NPs and no HA.

Given that realistic environmental concentrations of AgNPs are likely to be considerably lower (i.e. in the ng/L range), the effects of SRHA (and other sources of NOM) could have profound effects upon *L. variegatus* behaviour and as a result, their survival.

No detrimental effects upon behaviour were revealed following NM-104 exposure, indicating a lack of neurological damage as hypothesised for NM-300K or physical inhibition because of exoskeleton coating by NM-104 aggregates/agglomerates.

As a key macroinvertebrate, *L. variegatus* play an integral role within freshwater ecosystems. A lack of behavioural function because of NP exposure could result in increased *L. variegatus* predation, leading to changes within the nutrient cycle and food web which could ultimately lead to major ecological shifts. This research is the first to investigate the effects of AgNP exposure toward the locomotive behaviours of *L. variegatus* and only the second used in the context of NPs (O'Rourke *et al,* 2015 previously studied *L. variegatus* behaviour in relation to ZnO NP exposure). Similarly to O'Rourke *et al* (2015), locomotive assays were a valuable endpoint in assessing the toxic effects of NPs to *L. variegatus* within this research and should be considered as a useful biomarker in the ecotoxicological testing of NPs.

3.5 Conclusions

Although *L. variegatus* predominantly inhabits sediments, they are also capable of occupying the water column, potentially interacting with suspended contaminants. Screening NPs in the absence of sediment or a food source represents a worst-case exposure scenario for *L. variegatus.* It also provides an efficient and valuable source of ecotoxicological data. Acute aquatic exposures conducted within this investigation revealed the toxic nature of NM-300K towards *L. variegatus,* while NM-104 was found to be relatively non-toxic even at extremely high concentrations. The impact of abiotic factors (medium conductivity and NOM content) on NP toxicity within this investigation highlights the importance of environmental relevancy within NP ecotoxicology testing. Increased OECD medium conductivity (950 µS/cm) was found to significantly reduce NM-300K toxicity towards *L. variegatus* and whilst the inclusion of SRHA (5 mg/L) had no effect upon mortality, it proved to mitigate sub-lethal effects of NM-300K upon behavioural responses. Sub-lethal NM-300K exposures also revealed the ability of *L.*

variegatus to recover behavioural function following a period of behavioural decline. Body reversal and helical swimming responses measured within this investigation illustrate the value of behavioural assays as sensitive, non-destructive whole organism parameters of ecotoxicological testing.

Chapter 4: Investigation of NM-300K and NM-104 mediated oxidative stress in *L. variegatus.*

4.1 Introduction

Oxidative stress is amongst one of the primary toxic effects induced by NPs across mammalian cells and *in vivo* (rodent) models (Nel, 2006), and is a common consideration of hazard investigations when assessing the impact of NP exposure on human health and the environment. Oxidative stress is defined as the imbalance between oxidants and antioxidants in favour of oxidants, potentially leading to damage (Sies, 2000). NPs are able to generate reactive oxidant species (ROS) directly or stimulate their production within cells following exposure (figure 4.1).

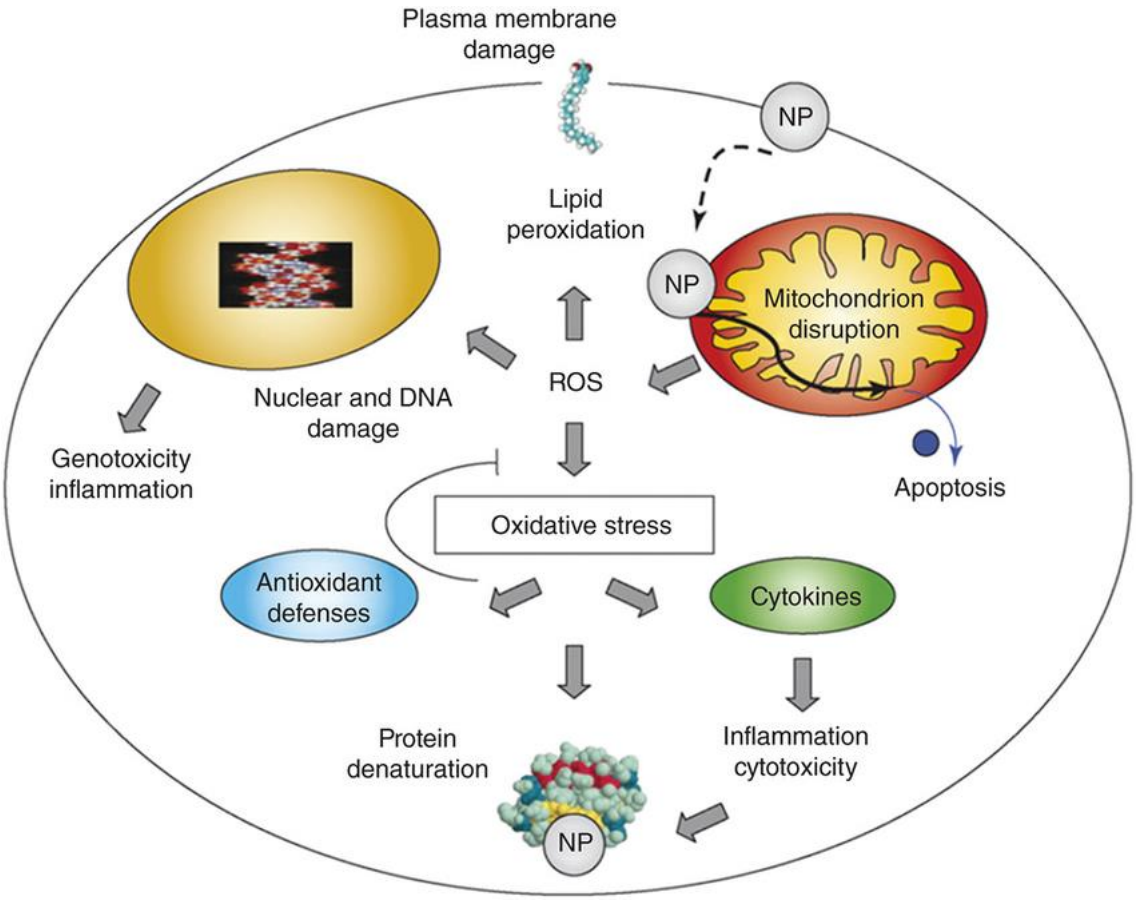

Figure 4.1. Nanoparticle- mediated production of reactive oxygen species and their potential biological responses. Source: Sanvicens and Marco, (2008).

Consequently, oxidative stress (monitored e.g. via antioxidant activity/level or damage to lipid, proteins and DNA) is frequently observed within NP toxicity assessments (Manke *et al,* 2013). AgNPs have been reported to mediate oxidative stress in numerous environmental species, including zebrafish (Choi *et al,* 2010), *Drosophila melanogaster* (Ahamed *et al,* 2009), *Caenorhabditis elegans* (Lim *et al,* 2012) and *Oryzias latipes* (Chae *et al,* 2009). Similarly, TiO_2NP exposure has shown to induce oxidative stress in *Oncorhynchus mykiss* (Federici *et al,* 2007), *Cyprinus carpio* (Linhua *et al,* 2009), zebrafish (Xiong *et al,* 2011) and *Caenorhabditis elegans* (Rui *et al,* 2013). However, data related to NP-mediated oxidative in *L. variegatus* is extremely limited with, to date no information in relation to AgNP or TiO_2NP.

4.1.1 Formation of Reactive Oxygen Species (ROS)

Free radicals are defined as a chemical species with one or more unpaired electrons, whilst the term 'reactive oxygen species encompasses a number of molecules (including free radicals and non-radical oxidising agents) derived from molecular oxygen (O_2) (Turrens, 2003). The majority of ROS are believed to be produced in the mitochondrion due to its pivotal role in aerobic metabolism (Turrens, 2003). It is speculated that between 1-2% of O_2 consumed as part of normal respiration is converted to superoxide radicals (Ott *et al,* 2007; Kehrer, 2000). ROS are maintained at a steady state concentration by a number of enzymatic and non-enzymatic antioxidants in cells. Antioxidants scavenge/detoxify ROS to protect cells from the damaging effects of ROS. ROS play a dual role in biological systems. Although slight fluctuations to oxidant/antioxidant homeostasis are thought to be involved in intracellular signalling processes (Dröge, 2002), an excessive imbalance between oxidant and antioxidants (in favour of the former) can lead to oxidative stress which can have deleterious effects on cell function.

During aerobic metabolism, O_2, (as the terminal electron acceptor) undergoes a four-electron reduction catalysed by the terminal enzymatic component, cytochrome-c oxidase, converting it to water. Although in its ground state, (triplicate) molecular oxygen is relatively unreactive, by accepting electrons the spin state is altered, increasing its reactivity and initiating the production of ROS. The initial excitation of

molecular oxygen also leads to the production of singlet oxygen, a very reactive oxidant (Turrens, 2003).

Superoxide anion ($O_2^{\bullet -}$) is the product of a one electron reduction of oxygen catalysed by the enzyme nicotinamide adenine dinucleotide phosphate–oxidase (NADPH oxidase). The subsequent dismutation of $O_2^{\bullet -}$, most notably by the antioxidant superoxide dismutase (SOD) produces O_2 and hydrogen peroxide (H_2O_2), which in turn can also be reduced by the antioxidant (catalase) to form one molecule of oxygen and two molecules of water (figure 4.1), or by peroxidases to form two molecules of water (Bayr, 2005, figure 4.2).

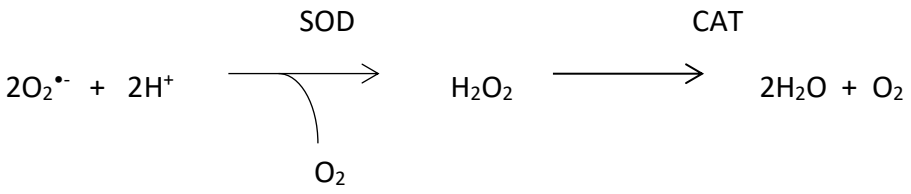

Figure 4.2 Dismutation of superoxide anion by superoxide dismutase and subsequent reduction of hydrogen peroxide by catalase.

Although $O_2^{\bullet -}$ and H_2O_2 represent relatively stable intermediates of oxygen reduction, they can chemically react with transition metals to generate the extremely toxic hydroxyl radical ($HO^{\bullet}$) through the Haber-Weiss reaction (figure 4.3). The production of $O_2^{\bullet -}$ and H_2O_2 as by-products of aerobic respiration reactions occurs within close proximity to large sources of redox iron (Fe) and copper (Cu) in the mitochondrial membrane, providing the ideal conditions for Fenton chemistry and subsequent $HO^{\bullet}$ production (Cadenas and Davies, 2000).

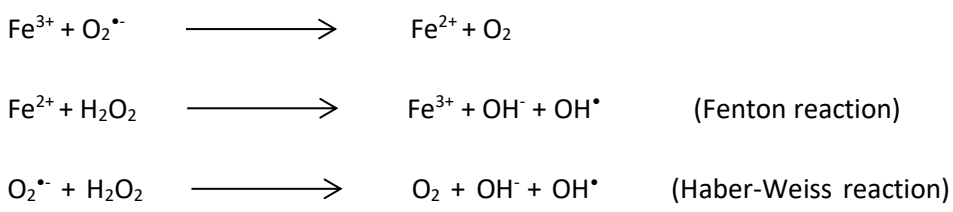

Figure 4.3. The Fenton reaction and Haber-Weiss reaction for the generation of hydroxyl radical.

The iron-catalysed Haber-Weiss reaction (using Fenton chemistry) is regarded to be the major route through which $HO^{\bullet}$ is generated in cells (Liochev and Fridovich, 1999). H_2O_2 plays a pivotal role in the production of $HO^{\bullet}$ and thus, the generation of the two are inevitably related (Cadenas and Davies, 2000). The hydroxyl radical is the most reactive oxidant and indiscriminately reacts with almost every component within living cells.

As $HO^{\bullet}$ can only diffuse short distances (several molecular distances) (Cadenas and Davies, 2000), and has a half-life of only 1 nanosecond or less (Pastor *et al,* 2000), it reacts with the first molecule it encounters, typically very close to its original site of generation. Although $HO^{\bullet}$ has a very short half-life and is extremely toxic, the two factors are not always intrinsically linked. For example, less toxic ROS with longer half-lives such as superoxide radicals can diffuse further away from their generation site (Sies, 1997) inflicting oxidative damage in more distant targets, including the mitochondrial genome (Kohen and Nyska, 2002). On the other hand, the toxicity of more reactive ROS with a shorter half-life is largely dependent on where they are produced as; if no biological target is available, no oxidative damage can occur.

4.1.2 Mechanisms of oxidative stress

The term 'oxidative damage' is described by Halliwell and Whiteman, (2004) as "*the biomolecular damage that can be caused by direct attack of reactive species during oxidative stress*". Lipid membranes, proteins and DNA are the main biological molecules to be targeted by ROS, and an increase in ROS production has been implicated in a plethora of diseases. To date, free radicals and ROS have been documented to be involved in the onset of a number of clinical human conditions; including but not limited

to diseases of the; brain, heart, lungs, kidney, gastrointestinal tract, red blood cells and eyes (Aruoma, 1998). Oxidative stress is also known to play an important role in carcinogenesis through DNA damage, (causing genetic mutations) and altering the expression of genes involved in cell growth (Klaunig and Kamendulis, 2004). The severity of injury sustained through oxidative stress is dependent on the molecular target, the mechanism through which ROS are generated and their concentration in relation to antioxidant enzymes

4.1.3 Lipid Peroxidation

Due to their high polyunsaturated fatty acid (PUFA) content, lipids are a prime target for ROS (and other free radical) attack via oxidation. The oxidation of a single lipid can stimulate a chain reaction whereby unsaturated lipids of a cell or organelle membrane become oxidised through the process of lipid peroxidation (Halliwell and Chirico, 1993). The first stage of lipid peroxidation, *'initiation'* involves a suitably reactive ROS, i.e. $HO^{\bullet}$ removing a hydrogen atom from the reactive methylene carbon (LH) of a PUFA side chain, leaving an unpaired electron on the carbon atom in the process. Consequently, a fatty alkyl free radical ($L^{\bullet}$) is formed (figure 4.4). PUFAs are particularly vulnerable to this form of reaction due to their high concentration of carbon double bonds (Halliwell and Chirico, 1993). In aerobic cells, the carbon centred radical $L^{\bullet}$ typically reacts with molecular oxygen to form a fatty peroxyl radical ($LOO^{\bullet}$).

$$OH^{\bullet} + LH \longrightarrow H_2O + L^{\bullet} \quad \text{(a)}$$

$$L^{\bullet} + O_2 \longrightarrow LOO^{\bullet} \quad \text{(b)}$$

Figure 4.4. Formation of fatty alkyl free radical ($L^{\bullet}$) from PUFA side chain and (b) the reaction of $L^{\bullet}$ with molecular oxygen to form fatty peroxyl radical ($LOO^{\bullet}$)

The second stage of lipid peroxidation involves the propagation of the chain reaction by $LOO^{\bullet}$ which are able to react with other fatty acid chains (as figure 4.3a) to form a lipid hydroperoxide ($LOOH^{\bullet}$) (figure 4.5).

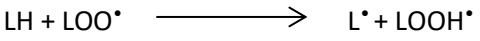

Figure 4.5. Reaction between fatty peroxl radical with fatty acid chains, forming a lipid hydroperoxide (LOOH•)

Consequently, the reaction of a ROS with a single PUFA can lead to an autocatalytic cycle whereby hundreds of PUFAs can be converted to lipid hydroperoxide. The length of propagation is dependent on intracellular oxygen concentrations, the protein content of membranes (which can be attacked by LOO• in place of fatty acids) and the presence of chain-breaking membrane antioxidants (most notably. α-tocopherol) (Haliwell and Chirico, 1993). Termination of lipid peroxidation occurs because of two radical species reacting with one another to produce a number of non-radical products (figure 4.6).

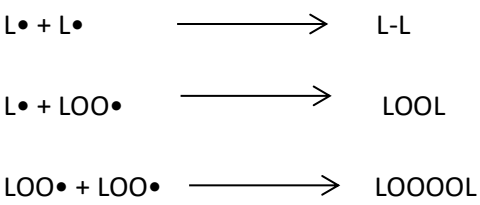

Figure 4.6 Reaction of two radical species, leading to the production of non-radicals and the termination of lipid peroxidation.

Lipid peroxidation can alter the structure of cell membranes, decreasing their fluidity and compromising their integrity (Slater, 1984), and organelle membranes (e.g. mitochondria), whilst the inactivation of membrane bound enzymes and receptors increase their permeability (Haliwell and Chirico, 1993), causing a loss of function. A wide range of damaging products are also produced through lipid peroxidation, including the endogenous genotoxic malondialdehyde (MDA) which has shown to damage DNA.

4.1.4 Antioxidant defences

The damaging effects of ROS can be offset by the action of both enzymatic (superoxide dismutase, catalase and glutathione peroxidase) and non-enzymatic (vitamins C and E, lipoic acid, uric acid, carotenes and ubiquinol) antioxidant defences. Whilst antioxidant enzymes are present at basal levels, cells can amplify their production and/or activity in response to increased oxidants.

4.1.5 Superoxide dismutase (SOD)

The ability of superoxide oxide dismutase (EC 1.15.1.1) to catalyse the dismutation of superoxide, was first discovered by McCord and Fridovich in 1969. The dismutation of superoxide by SOD involves the simultaneous removal or addition of electrons from/to superoxide molecules, producing O_2 and H_2O_2 (which can be decomposed by catalase) in the process. SOD is present in all aerobic organisms and in all subcellular compartments (e.g. mitochondria) where oxidants may become apparent (Van Breusegem *et al,* 1999).

Three distinct classes of SOD have been identified, each with metal cofactors; e.g. FeSOD, MnSOD and Cu/ZnSOD which are responsible for the dismutation of superoxide. FeSOD and MnSOD are both found in prokaryotes but may also be found in the chloroplast of certain plants (Van Camp *et al,* 1996) and the mitochondria of eukaryotes, respectively. Cu/ZnSOD is localised in the cytosol and extracellular components of eukaryotes. The biological importance of SOD has been highlighted through its ability to; limit mutation frequencies (Farr *et al,* 1986), and sensitivity to oxygen (Van Loon *et al,* 1986), improve immune responses (Parsad *et al,* 1994), increase resistance against damaging organic compounds (St. Clair *et al,* 1991) and extend life-spans (Melov *et al,* 2000; Orr and Sohal, 1994).

4.1.6 Catalase (CAT)

The decomposition of hydrogen peroxide by vegetable and animal extracts as witnessed by Schoenbein (1863) was originally considered to be a general capability of all enzymes

until Loew (1901) discovered it to be the role of a specialised enzyme, which he named catalase (EC 1.11.1.6). As a tetramer enzyme, catalase is composed of four identical monomers, each containing a heme prosthetic group at the catalytic centre (Boon *et al,* 2001) and is thought to be present in the peroxisones of nearly all aerobic cells. Although the mechanisms by which catalase catalyses the decomposition of H_2O_2 to form O_2 and H_2O are still not entirely apparent, it is generally considered to be a two-stage reaction.

Initially, a H_2O_2 molecule interacts with residues (His^{74} and Asn^{147}) within the heme group and is split due to the transfer of protons between its oxygen atoms. Consequently, one oxygen atom is removed and bound to the iron atom, whilst the remainder is released as water. During the second process, an additional H_2O_2 molecule interacts with the heme group and is split in a similar fashion and subsequently combines with the iron-bound oxygen to form H_2O and O_2 gas. This enzymatic reaction is speculated to be one of the most efficient within cells, with the ability to perform 200,000 catalytic events per second per subunit (Boon *et al,* 2001). Catalase has been shown to; prevent protein and DNA damage (Sinitsyna *et al,* 2006), enhance life-span; (Melov *et al,* 2000), (Orr and Sohal, 1994) and improve vitality (Jeulin *et al,* 1989), underlining its biological importance.

4.1.7 Nanoparticle-mediated oxidative stress

The production of ROS is thought to be the primary mode through which NPs exert toxicity in vertebrate and invertebrate models and has been witnessed for NPs of various physico-chemical properties. Manke *et al,* (2013) cite; NP- cell interactions, pro-oxidant functional groups and active redox cycling on the surface of NPs as crucial factors in NP mediated ROS production. As discussed previously, the small size of NPs results in a larger surface area, which in turn facilitates the display of molecules and atoms on the NP surface. Such structural changes influence the electronic properties of NPs and can lead to the creation of specific surface groups and reactive sites (Nel *et al,* 2006). The interaction of electron acceptor or donor active sites with molecular O_2 can lead to the formation of $O_2^{\bullet-}$, which can then enable the production of further ROS via dismutation and Fenton-type reactions (Nel *et al,* 2006, Manke *et al,* 2013).

ROS may also be generated when both oxidants and free radicals are present on NP surfaces. Ambient compounds such as ozone (O_3) and nitrogen dioxide (NO_2) are known to be capable of inducing ROS on the surface of NPs (Buzea *et al,* 2007). Transition metal NPs, including; iron, copper and chromium, are adept at the generation of ROS via Fenton-type (Buzea *et al,* 2007) and Haber-Weiss (Knaapen *et al,* 2004) reactions.

Mitochondria are key target for metallic NPs and consequently, the mitochondrial apoptotic pathway plays a major role in cell death because of NP mediated oxidative stress. The accumulation of NPs within mitochondria and the subsequent production of ROS may lead to the damaging of membrane phospholipids and can initiate mitochondrial membrane depolarisation (Lenaz, 2001). Through their interaction with the mitochondrial electron transport chain, xenobiotics, such as NPs are thought to enhance $O_2^{\bullet-}$ production by; blocking electron transport or accepting electron carriers from respiratory carriers, converting it to molecular O_2 via redox cycling (Turrens, 2003). The mechanisms through which ROS are generated are dependent on the NP in question and the cellular mechanisms are still not fully elucidated. Although oxidative stress is not a prerequisite for the mechanisms of NP toxicity, it is certainly considered a key paradigm and is an important parameter when considering their potentially harmful effects.

4.1.8 Ecotoxicological investigation of NP-mediated oxidative stress

Given that oxidative stress is reported to play a crucial role in NP toxicity, assessment of ROS production, the activity/level of antioxidant enzymes and detection of lipid peroxidation prove to be popular biomarkers within their ecological testing. Concentration dependent increases in CAT, glutathione peroxidase (GPX) and glutathione S-transferase (GST) were recorded for *D. magna* exposed to 5 and 10 mg/L TiO_2NPs (200-800 nm) for 48 hours, suggetsing the generation of ROS and development of oxidative stress, however no increases in SOD were detected (Kim *et al,* 2010). Hao *et al,* (2008) found the activity of SOD, CAT and peroxidase (POD) to be reduced in juvenile carp (*Cyprinus carpo)* over time (1-8 days) following exposure to higher concentrations of TiO_2 (100 and 200 mg/L, 50 nm), suggesting that the antioxidant defence system was potentially overwhelmed by the production of ROS. The

observation of elevated lipid peroxidation within the tissues further supported this theory.

Increasing levels of total glutathione and MDA (a by-product of cellular lipid peroxidation) were detected in the liver tissue of zebrafish exposed to AgNP (30-10 mg/L, 5-20 nm) for 24 hours (Choi *et al,* 2010). Depleted levels of catalase and glutathione mRNA and increased DNA damage also suggested the occurrence of oxidative stress and apoptosis. Similarly, Wu and Zhou (2012) revealed a AgNP (29.9 nm) concentration dependent (62.5 – 1000 µg/L) increase in SOD activity within the liver of Medaka (*Oryzias latipes*) across 9 days, along with increases in MDA content, suggesting that NPs mediated oxidative stress. Conversely, Farkas *et al,* (2010) detected no increased levels of ROS in the cells of rainbow trout exposed to AgNP at various concentrations (0.063 – 19 mg/L) for 2 hours, indicating that observed reductions in membrane integrity and cellular metabolic activity were not caused by oxidative stress.

Research regarding oxidative stress as a result of Ag and TiO_2 NP exposure is currently limited to freshwater invertebrates. Although oxidative stress assays have been applied to *L. variegatus* exposed to; herbicides (Cochón *et al,* 2007; Contardo and Wiegand, 2008; Contardo-Jara *et al,* 2009; Wiegand *et al,* 2006) and insecticides (Kristoff *et al,* 2008), their use in the testing of NPs is particularly limited. Wang (2014) found CAT activity to significantly increase in *L. variegatus* exposed to C_{60} fullerene NP spiked sediments for 14 days whilst O'Rourke *et al*. (2013, unpublished observations) detected no significant changes in *L. variegatus* glutathione levels following a 96-hour exposure to zinc oxide (ZnO) NPs. To date, no studies regarding Ag or TiO_2 NP-mediated oxidative stress could be identified for *L. variegatus.* Given the attention surrounding the ROS-generating capabilities of NPs and their potential environmental release, understanding the mechanisms through which they exert toxicity in environmentally pertinent species such as *L. variegatus* is crucial.

4.1.9 Aims

This study aims to infer what role oxidative stress has upon the sub-lethal toxicity of NM-300K and NM-104 towards *L. variegatus* across short-term, aquatic exposures via assessment of SOD and CAT. Lipid peroxidation of *L. variegatus* was also investigated to establish whether any oxidative damage was incurred as a direct result of either NM-300K or NM-104 exposure. The ability of SRHA to mitigate oxidative toxicity is also considered in regards to NM-300K. $AgNO_3$ exposures were included to determine whether the potential contribution of Ag dissolution to NM-300K mediated changes in antioxidant responses.

4.2. Methods

4.2.1 L. variegatus exposures for SOD and CAT analysis

Based on the toxicity data recorded in chapter 3, *L. variegatus* were exposed to sub-lethal concentrations of NM-300K (0.1 and 0.2 mg/L), NM-104 (2 mg/L) and $AgNO_3$ (10 µg/L) in OECD medium. A control of OECD medium was also included. A considerably lower concentration of $AgNO_3$ was selected given the higher toxicity observed in comparison to NM-300K (chapter 3). The effect of SRHA was investigated through the inclusion of 5 mg/L SRHA with 0.2 mg/L NM-300K in OECD medium. Preparation of NPs, SRHA and storage conditions were identical to those described in section 3.2.5. Three replicates (each containing 6 worms) were exposed to each treatment for 2, 6, 24, 48 and 72 hours.

4.2.2 L. variegatus sample preparation

Following exposure, worms were removed from vials, blotted dry on paper towels and weighed to ensure an appropriate amount of tissue mass (20-40 mg) was used for the antioxidant assays. Worms were subsequently transferred (via wooden pick) to 1.5 ml Eppendorf tubes containing 400 µL sodium phosphate buffer, pH 7, with 1 mM ethylenediaminetetraacetic acid (EDTA) and homogenised on ice using a small plastic spear until no large worm remains were visible. Samples were centrifuged at 500 g, 4°C for 15 minutes, after which the supernatant was removed and transferred to a fresh Eppendorf tube, which was then centrifuged at 12,000 g, 4°C for 45 minutes. The supernatant was then transferred to a fresh Eppendorf tube and kept on ice before analysis.

4.2.3 SOD assay

SOD activity of *L. variegatus* was determined using a 96-well plate adapted from Del-Maestro and McDonald (1985). The principal of the assay is based on the ability of SOD to scavenge $O_2^{\bullet -}$, which in turn, shortens reaction chains and inhibits the auto-oxidation of the organic compound, pyrogallol. Reagents used in the assay were made fresh on the day of testing and included:

- 50 mM tris-HCl buffer containing 1 mM diethylene triamine pentaacetic acid (DTPA). Following preparation, the solution was stirred at room temperature for ~20 minutes to equilibrate, after which pH was adjusted to 8.2 using 1M HCl.
- Pyrogallol stock (20 mM) – made in 10 mM HCl.

Following preparation, 20 µL of each *L. variegatus* homogenised tissue sample and controls (20 µL dH_2O – no *L. variegatus* tissue) were added to the wells of a clear, flat bottomed 96-well plate. Immediately before performing the assay, a 1 in 100 dilution of pyrogallol stock in 50mM tris-HCl buffer was made, 180 µL of which was added to each well, giving a final volume of 200 µL. The optical density was measured at 420 nm for 3 minutes (with readings taken every 10 seconds) using the Molecular Devices SpectraMax® M5 microplate reader. The protein content of test samples was measured using the Bradford assay (section 4.2.5). SOD content was determined as follows:

$$ng\ SOD\ per\ mg\ protein = \frac{\%\ INHIB}{50} \times 125 \times 0.2 \times \frac{1000}{S_v} \times \frac{1}{PROT}$$

Where:

- % INHIB = inhibition of pyrogallol autooxidation caused by samples in comparison with controls (20 µg/L dH_2O) - determined by change in OD at 420 nm for 3 minutes.
- 50 = the % at which one unit of SOD inhibits autooxidation.
- 125 = 50% inhibition is represented by 125 ng/ml purified bovine CuZn SOD (Del-Maestro and McDonald, 1986).
- 0.2 = volume in each well (ml).
- S_v = sample volume (µL)
- PROT = protein content of sample per ml as measured by the Bradford protein assay (section 4.2.5).

4.2.4 CAT assay

CAT activity was determined from a protocol adapted from Claiborne (1985) and Wang (2012), based on the ability of CAT to decompose H_2O_2. Reagents used in the assay included:

- 50 mM potassium phosphate (in dH_2O), pH7.
- 3% H_2O_2 – 30% H_2O_2 (Sigma-Aldrich®) diluted 1 in 10 with 50 mM potassium phosphate pH 7.

To the wells of a clear, flat bottomed UV 96-well plate, 180 µL of 50 mM potassium phosphate buffer and 50 µL of homogenised *L. variegatus* sample were added. H_2O_2 (3%, 20 µL) was then added to each well, after which the absorbance at 240 nm was read on a SpectraMax® M5 microplate reader for 4 minutes (readings taken every 10 seconds). The protein content of test samples was measured using the Bradford assay (section 4.2.5). CAT activity was expressed as specific activity and determined as follows:

$$\text{Specific activity (units per mg)} = \frac{\Delta A \times 1000}{43.67 \times mg\ protein\ per\ ml\ reaction\ mixture}$$

Where:

- ΔA is the difference of UV absorbance reading over 4 minutes.
- 43.67 represents the molar coefficient of H_2O_2 in the reaction at 240 nm.
- Protein content of sample (mg/ml) as calculated by Bradford protein assay (section 4.2.5).

4.2.5 Bradford protein assay

L. variegatus protein content was determined for both SOD and CAT assays using the Bradford assay. Bovine serum albumin (BSA, Sigma-Aldrich®) was used as a standard in the assay. BSA standards were made in dH_2O from a 1 mg/ml stock to give

concentrations of; 0, 0.03125, 0.0625, 0.125, 0.250, 0.5 and 1 mg/ml which were made up fresh and stored on ice until use. *L. variegatus* tissue samples (as prepared in section 4.2.2) were diluted 1 in 50 with dH_2O and kept on ice. 10 µL of each standard or sample was transferred in triplicate to the wells of a clear bottomed 96-well plate, to which 200 µL of BioRad protein assay dye reagent (diluted 1 in 4 with dH_2O) was added to give a final volume of 210 µL per well. Plates were then read and the protein content of each sample calculated (from the standard curve) in µg/ml.

4.2.6. Principal of TBARS assay

Lipid peroxidation was assessed using the BioAssay Systems QuantiCrom™ TBARS assay kit. The assay is based on the reaction of TBARS (low molecular weight end products, predominantly MDA, formed during the decomposition of lipid peroxidation products) with thiobarbituric acid (TBA) to form a pink coloured product. The optical density (OD) at 535 nm of said product is directly proportional to the TBARS concentration in the sample and thus, the level of lipid peroxidation.

4.2.7 TBARS exposures and sample preparation

L. variegatus were exposed in OECD medium containing; 0.1, 0.2 mg/L NM-300K, 0.2 mg/L NM-300K in the presence or absence of 5 mg/L SRHA or 2mg/L NM-104. OECD medium containing no NPs were included as a control. Preparation of NPs, SRHA and storage conditions were identical to those described in section 3.2.5. Three replicates of six worms were exposed for 24 and 48 hours to each test substance described above. Following exposure, worms were; blotted dry, weighed (20-40 mg tissue per replicate) and homogenised in 200 µL ice cold phosphate-buffered saline (PBS) in a 1.5 ml Eppendorf tube using a plastic spear. Once all tissue was homogenised, 200 µL of 10% trichloroacetic acid (TCA) was added to 100 µL of each sample and incubated on ice for 5 minutes. Samples were then centrifuged for 5 minutes at 14,000g after which 200 µL of the supernatant was transferred to new Eppendorf tube.

4.2.8 TBARS Assay

TBA (Thiobarbituric Acid) reagent (200 µL) was added to each of the samples and standards (table 4.1), which were vortexed and subsequently incubated on a heat block at 100°C for 1 hour. Eppendorfs were then left to cool to room temperature, vortexed and centrifuged at 14, 000g for 2 minutes before 100 µL of each standard and sample was loaded to the wells of a clear-bottomed 96-well plate in duplicate. Absorbance was read at 535 nm using the SpectraMax® M5 microplate reader.

Table 4.1 MDA standard preparation for TBARS assay

Standard No.	30 µM MDA + H2O	Volume (µL)	MDA (µM)
1	300µL + 0µL	300	30
2	180µL + 120µL	300	18
3	90µL + 210µL	300	9
4	0µL + 300µL	300	0

The standard blank (standard number 4, table 4.1) OD value was subtracted from each standard and sample OD. The ΔOD_{535} was plotted against standard concentrations to determine the slope of the standard curve. TBARS concentration of samples was determined using the following equation:

$$TBARS\ (\mu M\ MDA\ equivalents) = \frac{OD_{sample}\ -\ OD_{blank}}{Slope\ (\mu M^{-1})} \times 3\ (dilution\ factor)$$

4.2.9 Statistical analysis

Data was assessed for normality and homogeneity of variance using a Shapiro-Wilk test. Two-way ANOVA analysis and Tukey tests were used to determine whether NP concentration (and the inclusion of SRHA) or time were significant in determining *L. variegatus* SOD levels or CAT activity following NM-300K or NM-104 exposure. Individual one-way ANOVA and corresponding Tukey tests were performed for each timepoint to determine whether different NM-300K concentrations (and the inclusion of SRHA) had any significant influence ($p<0.05$) on SOD levels or CAT activity. One-way ANOVA and Tukey tests were also used to detect significant differences ($p<0.05$) between SOD levels and CAT activity for *L. variegatus* exposed to 0.2 mg/L NM-300K over the 2-72 hour period. All statistics performed in IBM SPSS 22.

4. 3 Results

4.3.1. NM-300K SOD and CAT

NM-300K concentration and length of exposure were found to significantly influence *L. variegatus* content (two-way ANOVA, $p<0.01$). Individual one-way ANOVA and Tukey tests for each time-point detected significant differences between NM-300K concentrations ($p<0.05$) after 2, 24 and 48 hours (figure 4.7).

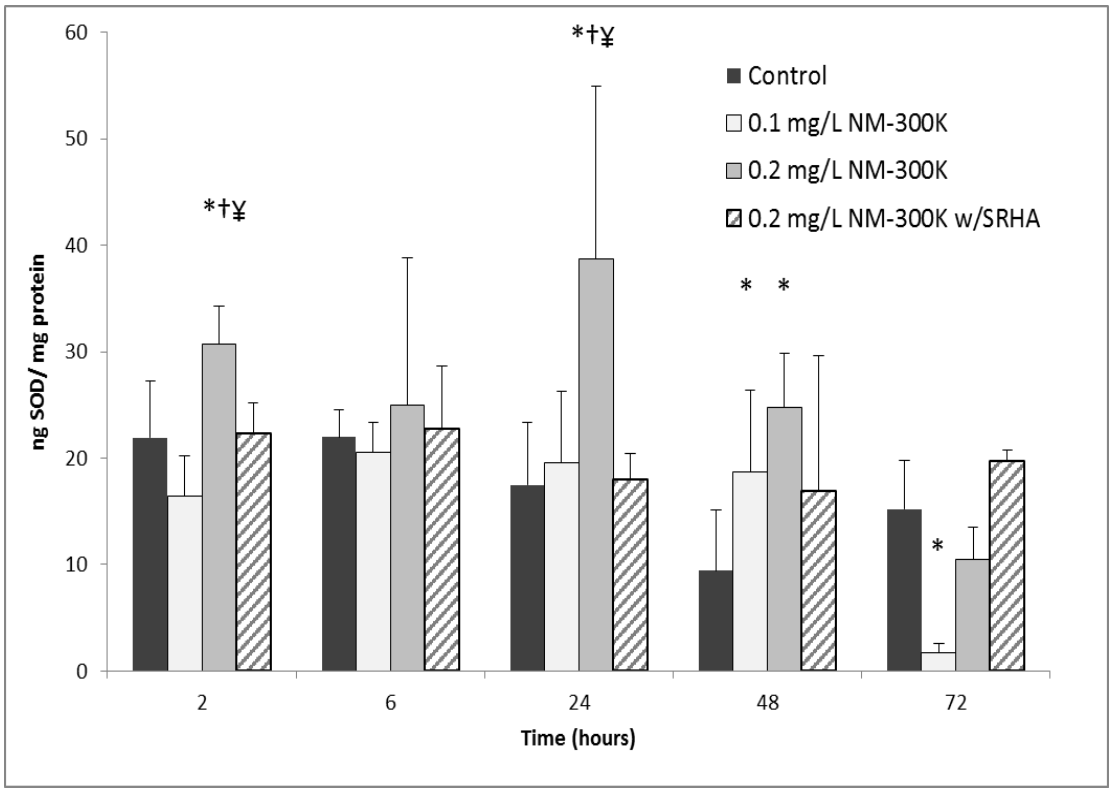

Figure 4.7 Mean SOD content of *L. variegatus* exposed to; 0 (OECD medium), 0.1 and 0.2 mg/L NM-300K (with and without SRHA) between 2-72 hours in OECD medium. Significant difference from control (*), 0.1 mg/L NM-300K (†) and 0.2 mg/L NM-300K w/SRHA (¥). Data expressed as mean +SD (n=3).

Exposure to 0.1 mg/L NM-300K only evoked a significant increase in SOD content (in comparison to controls) following a 48-hour exposure. Worms exposed to 0.2 mg/L NM-300K for 2 and 24 hours recorded significantly higher ($p<0.05$) levels of SOD content than all other samples, whilst after 48 hours, recorded higher levels than control worms only. No significant changes in SOD content were observed for 0.2 mg/L NM-300K at 6 or 72 hours. Worms exposed to 0.2 mg/L NM-300K in OECD medium containing 5 mg/L SRHA experienced no elevated levels of SOD across all time points tested and were

found to be significantly lower than 0.2 mg/L exposed worms in OECD medium containing no SRHA after 2 and 24 hours.

Similarly to SOD, both NM-300K concentration and exposure time were found to significantly influence *L. variegatus* CAT activity (two-way ANOVA, $p<0.05$). However, individual one-way ANOVA and tukey tests only for each timepoint only detected significant differences after 24 hours (figure 4.8). Worms exposed to 0.2 mg/L NM-300K were found to have significantly greater CAT activity than all other samples tested at 24 hours. No significant changes in CAT activity were observed in *L. variegatus* exposed to 0.1 mg/L NM-300K in comparison to controls across all time points. In the presence of SRHA, CAT activity was similar to control *L. variegatus* across all time points.

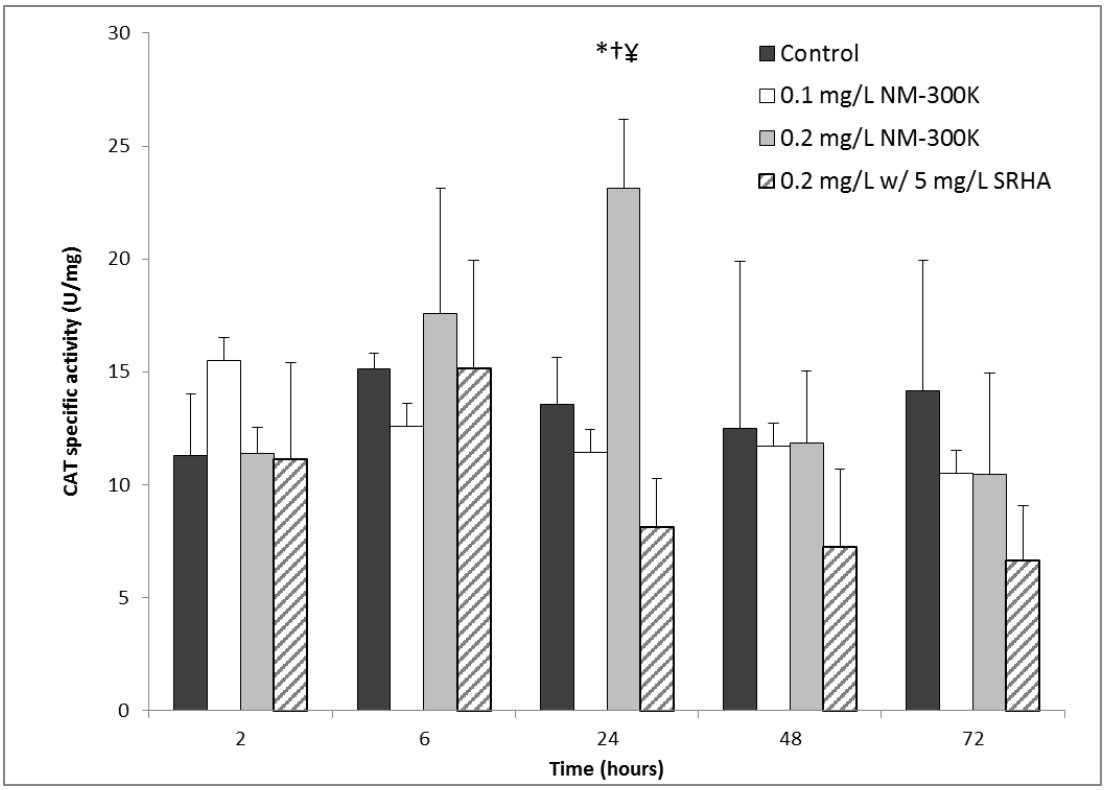

Figure 4.8 Mean CAT specific activities of *L. variegatus* exposed to; 0 (OECD medium), 0.1 and 0.2 mg/L NM-300K (with and without SRHA) between 2-72 hours in OECD medium. Significant difference from control (*), 0.1 mg/L NM-300K (†) and 0.2 mg/L NM-300K w/SRHA (¥). Data expressed as mean +SD (n=3).

Both SOD and CAT results of 0.2 mg/L-exposed worms displayed comparable behaviour over time (figure 4.9). Despite an initial decline of activity after 6 hours for SOD, the activity of both antioxidants peaked after 24 hours (both of which were found to be

significantly different from their corresponding readings at 2, 48 and 72 hours). Following 48 hours, levels of SOD and CAT activity returned to levels similar to those experienced at 2 and 6 hours. Further decline in both SOD and CAT was evident up to 72 hours, both of which were found to be significantly different from their corresponding readings after 6 hours.

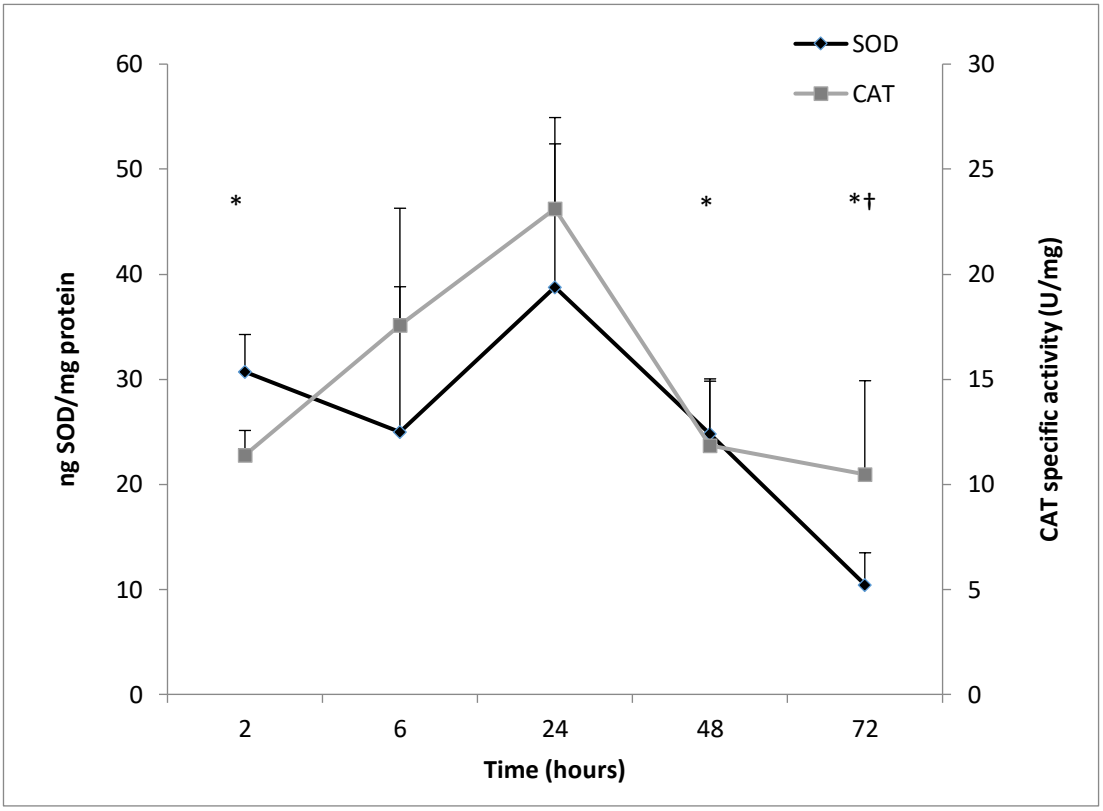

Figure 4.9 Mean SOD content and CAT activity of *L. variegatus* exposed to 0.2 mg/L NM-300K for 2, 6, 24, 48 and 72 hours. Significant difference from 24 (*) and 6 hours (†) ($p<0.05$). (+SD).

4.3.3 $AgNO_3$ SOD and CAT

L. variegatus exposed to 10 µg/L $AgNO_3$ in OECD medium showed no significant increases in CAT activity or SOD content compared to those exposed in control conditions for 2 and 24 hours (figure 4.10A and B).

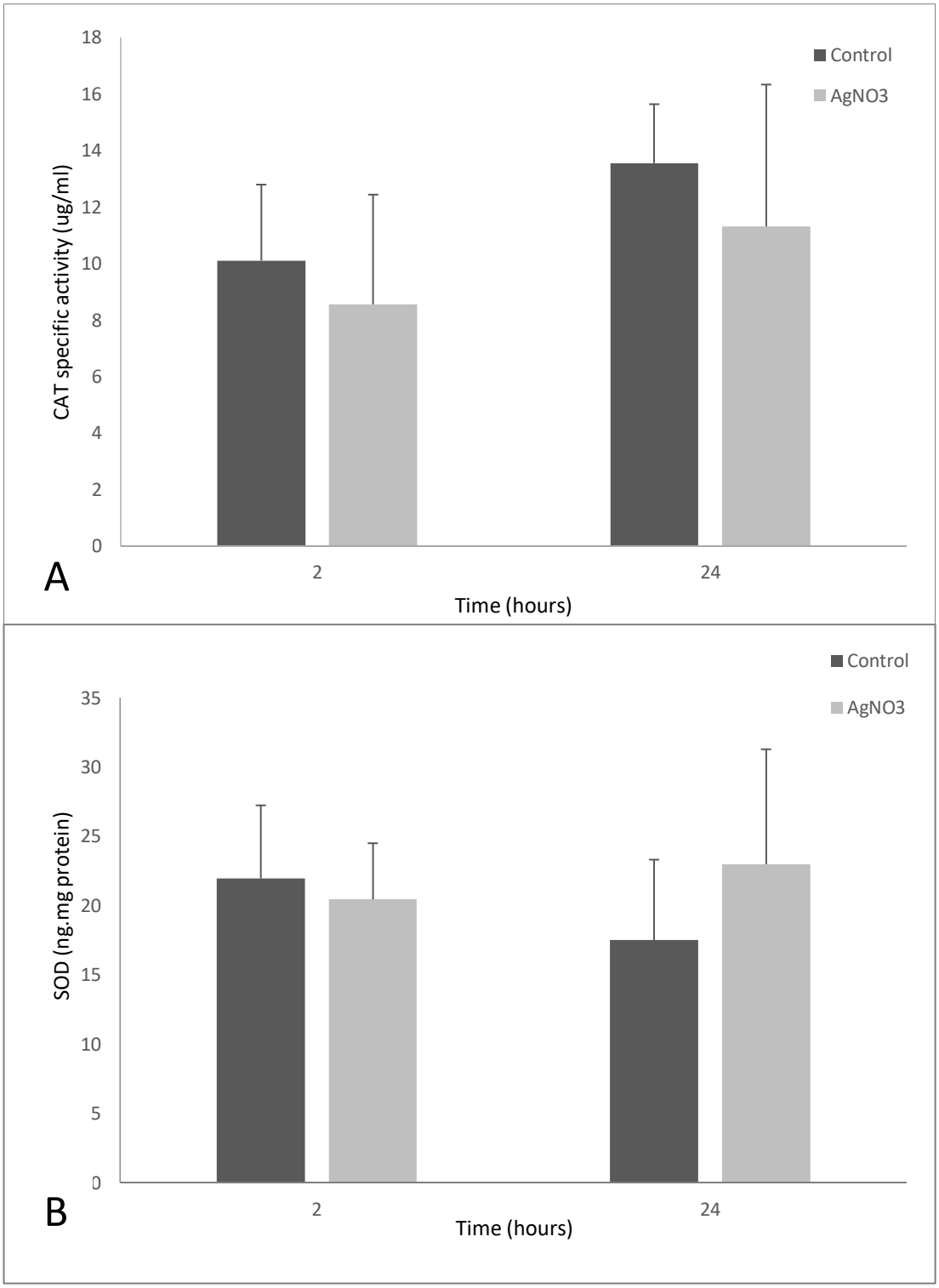

Figure 4.10. Mean CAT specific activity (A) and SOD content (B) of *L. variegatus* exposed to 0 (OECD medium) and 10 µg/L $AgNO_3$ in OECD medium for 2 and 24 hours. Data expressed as mean +SD (n=3).

4.3.4 NM-104 SOD and CAT

Two-way ANOVA analysis detected no significant changes in *L. variegatus* exposed to NM-104 across the 2-72-hour time period in comparison with controls (figure 4.11). Although a decline in SOD content following 24 hour exposure to NM-104 was observed, a one-way ANOVA and tukey test detected no significant difference from the control ($p<0.05$). Two-way ANOVA analysis found NM-104 to significantly influence the CAT activity of *L. variegatus.* Individual one-way ANOVA and Tukey tests detected a significant ($p<0.05$) decline in CAT activity after 24 hours, in comparison with controls (figure 4.12).

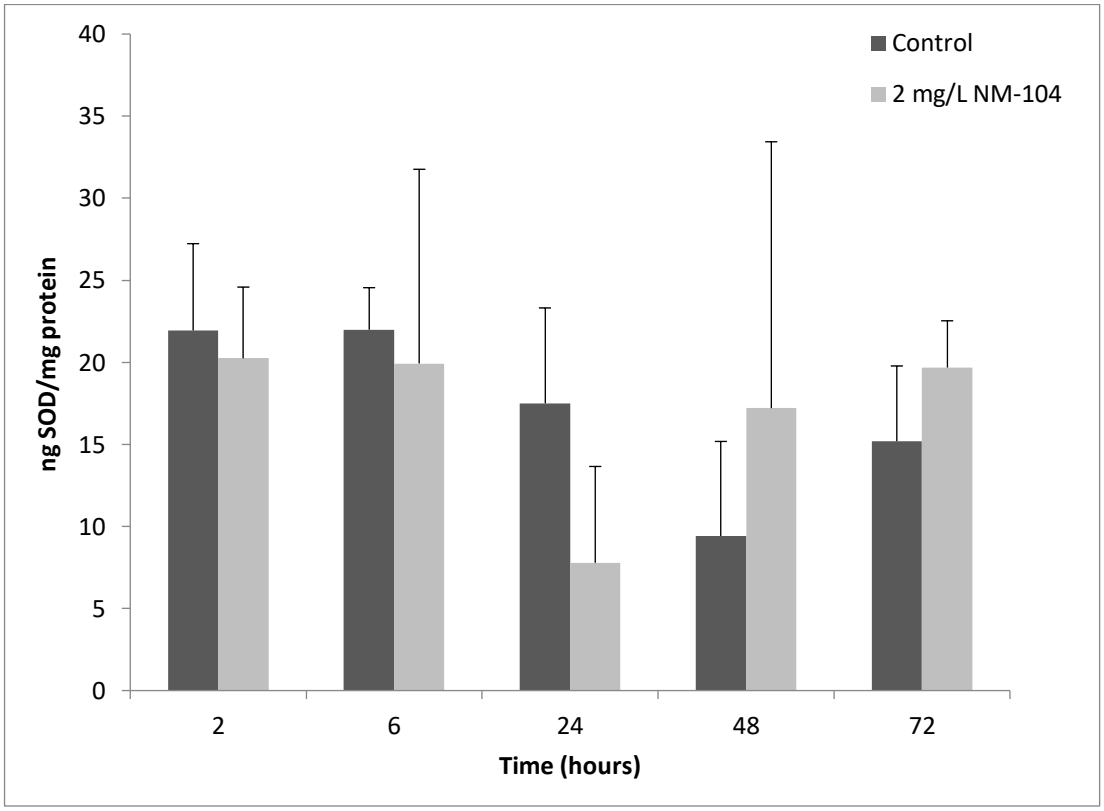

Figure 4.11. Mean SOD content of *L. variegatus* exposed to 0 (OECD medium) and 2 mg/L NM-104 for; 2, 6, 24, 48 and 72 hours. (+SD).

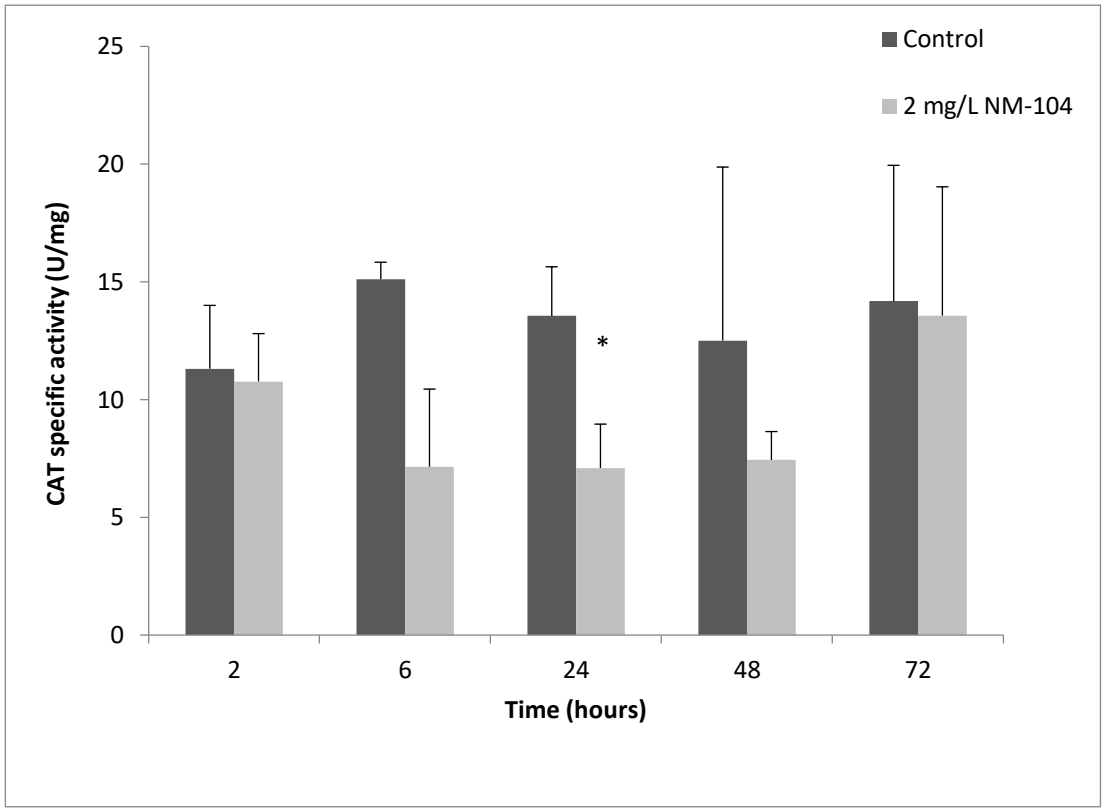

Figure 4.12 Mean CAT activities of *L. variegatus* exposed to 0 (OECD medium) and 2 mg/L NM-104 for 2, 6, 24, 48 and 72 hours. * denotes significant difference from control ($p<0.05$). Data expressed as mean +SD (n=3).

4.3.2 Lipid Peroxidation

No significant changes in lipid peroxidation were observed for NM-300K or NM-104 after 24 or 48 hours compared with controls ($p>0.05$) (figure 4.13).

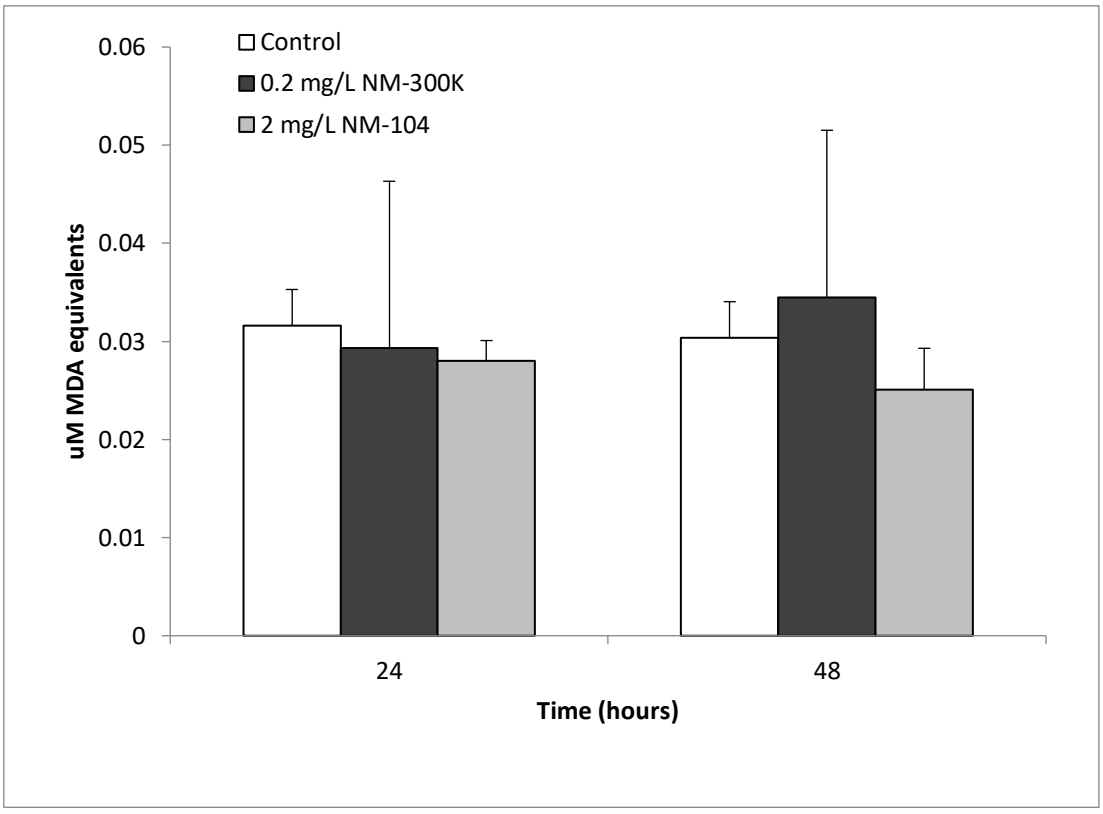

Figure 4.13 Mean MDA equivalents for *L. variegatus* exposed to OECD medium only (control), or 0.2mg/L NM-300K or 2 mg/L NM-104 for 24 and 48 hours. Data expressed as mean +SD (n=3).

4.4 Discussion

4.4.1 Antioxidant enzymes

As highlighted by Livingstone (2001), the regulation of antioxidant systems is poorly understood for aquatic species in relation to either the endogenous or exogenous sources of ROS (Livingstone, 2001). The results of this research suggest that NM-300K (at sub-lethal concentrations) can induce oxidant-driven responses in *L. variegatus*, as indicated by a rise in antioxidant activity. It is possible that the action of SOD and CAT could protect against oxidative damage (in the form of lipid peroxidation) in *L. variegatus* exposed to 0.2 mg/L NM-300K, however, future studies investigating additional parameters of oxidative damage (e.g. to DNA) and time points could be investigated to conform this. These findings align with the observation that aquatic species, due to their heightened sensitivity to chemical exposure and toxicity can provide a useful insight into the subtle effects of oxidative stress (Lackner, 1988). The mitigating effect of humic acid was demonstrated with no significant changes to SOD and CAT observed. No significant changes in SOD content were recorded for NM-104 across the exposure time-periods tested, while CAT activity remained relatively unchanged (except for 24 hours, where a significant decline was recorded).

The antioxidant enzymes of aquatic species are known to be rather sensitive or transient, e.g. to seasonal changes (Gabryelak *et al,* 1983) and feeding behaviour (Radi *et al,* 1985). In this study both SOD content and (to a greater extent) CAT activity were consistently similar throughout control samples over the course of the 72-hour experiment. It can be therefore assumed that the increases witnessed for SOD content and CAT activity following 0.2 mg/L NM-300K exposure were a consequence of AgNP-mediated oxidative stress alone and not related to; culture conditions (oxygen, food or light supply, temperature etc.), transfer during experimental set-up or an absence of food during exposure.

Though certain studies witness a depletion of antioxidant enzyme content/activity in freshwater species as a result of nanoparticle exposure (Hao *et al,* 2008), (Choi *et al,* 2010), it is important to emphasise that responses are highly variable between species and the contaminants to which they are exposed. The transient nature of antioxidant enzymes following the onset of oxidative stress must also be taken into consideration

as ROS generation and oxidative stress occur as early events of NP-induced toxicity (Manke *et al,* 2013).

As the first line of defence against oxidative stress, it is plausible that after an initial increase in SOD and CAT content/activity that either one or both may reach a saturation point, after which a depression or inhibition of activity is experienced as the antioxidants become overwhelmed. Similar findings have been reported in relation to the dose of toxicants administered, whereby low doses of contaminants such as; atrazine (Song *et al,* 2009), TiO_2NP and ZnONPs (Hu *et al,* 2010) have evoked increases in the SOD and CAT activities of the earthworm, *Eisenia fetida*, whilst higher concentrations have stimulated an inhibition. However, these findings may be time-dependent, i.e. at higher contaminant concentrations, antioxidants may become overwhelmed and eventually decline, taking time to return to control levels. By testing more than one NP concentration (for NM-300K) across numerous exposure times (including 3 early time points of 2, 6, and 24 hours), this investigation attempted to compensate for the transient nature of SOD and CAT.

The interdependent relationship between SOD and CAT was evident for *L. variegatus* exposed to 0.2 mg/L NM-300K. Both enzymes appeared to peak after 24 hours, following which a decline was observed. A similar pattern between the two enzymes can be attributed to the fact that CAT is responsible for the decomposition of H_2O_2, a by-product of $O_2^{\bullet-}$ dismutation. Whilst SOD experienced a steady decline after 48 and 72 hours, the decline in CAT activity proved to be less severe, suggesting a continued presence of H_2O_2 as a result of earlier SOD activity. The return of SOD and CAT to normal levels (i.e. similar to that of controls) after 48 and 72 hours suggest that at 0.2 mg/L NM-300K, *L. variegatus* antioxidant defence system are able to offset the actions of oxidative stress. Although some continued depletion was evident after 72 hours, indicating potential enzyme saturation, these readings were still found to be significantly similar to those of controls. Exposure periods beyond 72 hours would have to be investigated to ascertain whether depletion would continue or if levels would remain consistent with those of the controls.

After recording significantly higher recordings for multiple time periods (2, 24 and 48 hours) it can be concluded that SOD was a more sensitive biomarker than CAT (which was only found to be significantly greater after 24 hours) following 0.2 mg/L NM-300K

exposure. Contardo-Jara *et al,* (2009) also found SOD to be a more sensitive biomarker than CAT in *L. variegatus* exposed to glyphosate.

Both CAT activity and SOD content of *L. variegatus* exposed to 10 µg/L $AgNO_3$ remained comparable to that of control *L. variegatus*. Studies conducted in section 2.3.4, found dissolution of Ag^+ from NM-300K in OECD medium after 24 hours (the time period after which the greatest elevation in SOD and CAT were observed in 0.2 mg/L exposed worms) to be <3%. Based on these findings, a concentration of 0.2 mg/L NM-300K in OECD medium would lead to a dissolved Ag^+ concentration of approximately 8 µg/L. As no changes in SOD or CAT were observed following 24-hour exposure to 10 µg/L $AgNO_3$ (a concentration marginally higher than anticipated dissolution), it is possible to elucidate that Ag^+ has a negligible effect in relation to the oxidative stress parameters tested and that the results observed can largely be attributed to NPs alone.

The SOD content and CAT activity of *L. variegatus* exposed to 2 mg/L NM-104 remained similar to that of controls throughout with the exception of CAT after 24 hours. These results suggest that either;

- NM-104 did not induce the production of ROS and hence, *L. variegatus* had no requirement for an increase in antioxidant defences.
- That an initial increase in SOD and CAT went undetected (i.e. potentially increased outside of the time points selected) after which both enzymes stabilised.
- Higher concentrations of NM-104 are required to induce oxidative stress-mediated toxicity.

In relation to the anticipated concentrations of TiO_2NPs in the environment, the concentration employed within this investigation (2 mg/L) are somewhat unrealistic. Although greater NM-104 concentrations could be employed to investigate oxidative stress parameters (i.e. SOD and CAT), this would represent a greater deviation from predicted environmental concentrations. Furthermore, previous studies (chapter 3) recorded no toxicity in *L. variegatus* (in relation to mortality and behaviour) exposed to concentrations of NM-104 up to 1.5 g/L. The results obtained within this investigation suggest that NM-104 does not induce the production of ROS – further underlining its non-toxic nature towards *L. variegatus.*

The highly photoactive nature of TiO_2NP facilitates the production of ROS upon illumination (Jovanović, 2015). A light: dark cycle of 16:8 hours was maintained for all exposures and thus, any NM-104 ingested by *L. variegatus* is likely to have undergone photoactivation – however, no indicators of oxidative stress were observed. By varying the UV irradiation or light wavelength to which NM-104 is exposed could be used to assess the role of photoactivation on the oxidative stress capabilities of NM-104 toward *L. variegatus.*

4.4.2 Comparison with similar antioxidant studies

The investigation of *L. variegatus* antioxidant enzymes in relation to other, non-NP contaminants have produced similar results to those obtained for NM-300K as part of this investigation. Contardo-Jara and Wiegand, (2007) witnessed an increase in CAT activity following exposure to contaminated sediment and atrazine after 4 hours, following which significant depletions were observed over a 7-day period. A concentration dependent increase in SOD and CAT content/activity was also recorded by Contardo-Jara *et al* (2009) after exposure to glyphosate and its formulation, Roundup Ultra. After observing depleted activity of *L. variegatus* SOD and CAT after 48 and 96 hours of exposure to Azinphos-methyl, Kristoff *et al,* (2008) speculate that an anticipated upregulation was not observed due to the damaging effects of ROS which inhibited antioxidant enzyme activity. An increase in *L. variegatus* CAT activity as a result of carbon fullerene (C_{60}) exposure (Wang *et al,* 2014) represents, to date the only *L. variegatus* antioxidant enzyme study in relation to NPs.

Aside from *L. variegatus,* alternate freshwater oligochaete species, namely, *Tubifex tubifex* has demonstrated an increase in CAT activity upon exposure to a number of substances, including; chitosan (Mosleh *et al,* 2007), the fungicide fenhexamid (Mosleh *et al,* 2005), the herbicide isoproturon (Mosleh *et al,* 2005) copper (Mosleh *et al,* 2006) and chlorine (Nie *et al,* 2010). Similarly, Arendarczyk *et al* (2014) also recorded an increase in *T. tubifex* CAT activity after 24 hours' exposure to cadmium. Increases in SOD activity have been reported for terrestrial oligochaetes; *Enchytraeus albidus* following exposure to copper (Howcroft *et al,* 2009) CuNP (Gomes *et al,* 2012) and zinc (Novais *et al,* 2011) contaminated soils and *Eisenia fetida* following exposure to cadmium (Zhang

et al, 2009) and ZnO NP (Li *et al,* 2011). The expression of SOD and CAT genes in *E. fetida* have also been found to be upregulated following exposure to both AgNP and $AgNO_3$ contaminated soils (Hayashi *et al,* 2013).

During this study, *L. variegatus* SOD and CAT activity proved to be related to one another as well as the concentration and duration of NM-300K exposure. It is therefore recommended that the impact of NP exposure on antioxidant defences of *L. variegatus* are routinely investigated within ecotoxicology.

4.4.3 Role of NOM on antioxidant enzymes.

Although NOM substances have been reported to directly induce adverse effects upon freshwater species via the external and internal production of ROS (Paul *et al,* 2004; Timofeyev *et al,* 2004), the results of this investigation suggest that humic acid had a mitigating effect upon NM-300K mediated oxidative stress towards *L. variegatus* (despite the limitation of no SRHA control). CAT activity and SOD content of worms exposed to 0.2 mg/L NM-300K in OECD medium containing 5 mg/L SRHA were found to be comparable to those of controls, whilst in the absence of SRHA, 0.2 mg/L NM-300K induced significant increases in SOD and CAT.

Despite the popular consensus that humic substances may adversely affect organisms via oxidative stress, Steinberg *et al,* (2007) held the view that they are potentially able to transcriptionally control antioxidant and anti-stress enzyme activities in order to extend lifespan. By exposing the free-living soil nematode, *C. elegans* to humic substances, Steinberg *et al,* (2007) found certain 'anti-stress' genes to be either upregulated (in the case of superoxide dismutase) or down regulated (in the case of catalase) in a twofold manner in comparison with controls. Furthermore, catalase can become trapped within humic macromolecular structures to form stable humic-enzyme associations which have shown to retain a considerable portion of enzyme activity (Serban and Nissenbaum, 1986).

Although the aforementioned interactions between SRHA and antioxidant enzymes are a possible explanation for the results obtained in this investigation, they remain unlikely. The formation of a SRHA-CAT complexing suggests an inhibition of CAT and thus, a

potential increase in oxidative stress and subsequent organism damage. However, SRHA was previously found to reduce NM-300K toxicity (chapter 3). Using the same time-points selected for the antioxidant assays (2-72 hours), *L. variegatus* exposed to 0.2 mg/L NM-300K in 5 mg/L SRHA OECD media experienced no adverse changes in behavioural responses suggesting no lasting damage had been incurred. However, severely diminished behavioural responses were recorded for *L. variegatus* exposed to the same concentration in the absence of SRHA. CAT activity and SOD content of *L. variegatus* exposed to NM-300K in SRHA were similar to those of control *L. variegatus,* suggesting that no up or down regulation of either gene had occurred in the time frame tested.

The coating of AgNPs with NOM is known to limit dissolution rates (Liu and Hart, 2010) and is thus thought to be one of the major contributing factors in the mitigation of NP toxicity by NOM (Gao *et al,* 2012). However, within this study, 5mg/L SRHA was found to have no effect upon NM-300K dissolution (section 2.3.4), suggesting that NOM inhibited nano-specific toxicity of NM-300K towards *L. variegatus*. The mechanisms through which NOM mitigates NP toxicity is unclear. In light of the dissolution data collected within this investigation, it is more likely that SRHA limited NM-300K toxicity either by simply providing a physical barrier between NP-cell interactions or acting as an antioxidant through reactions with ROS (potentially) produced by NM-300K.

Although zeta potential data suggested little NOM coating of NM-300K particles occurred, (section 2.3.1), TEM images revealed large filamentous structures (hypothesised to be decaying vegetation) and dark, globular-like structures (also presumed to be a component of SRHA), to which NM-300K particles were attached and incorporated, as confirmed by EDS, (section 2.3.3). It is possible that these structures went undetected in DLS recordings, leading to misleading results in relation to NOM coating of NM-300K. A proposed SRHA-mediated reduction in ROS would limit the need for antioxidant enzymes such as SOD and CAT and is thus also a plausible explanation for the results obtained within this investigation (however further studies into ROS production both in the presence and absence of SRHA would have to be implemented to confirm this).

4.4.4 Lipid peroxidation

Despite incidents of elevated SOD and CAT content/activity as a result of NM-300K exposure, indicating that NPs may induce toxicity via oxidant driven mechanisms, no evidence of lipid peroxidation (as assessed by the TBARS assay) was detected for *L. variegatus* using the same concentration and exposure conditions. As with CAT and SOD, no publications addressing *L. variegatus* lipid peroxidation in relation to AgNP or TiO_2NP exposure are currently available. However, the results of this investigation concur with the findings of Cochón *et al,* (2007) who, despite finding transient changes to *L. variegatus* CAT activity as a result of paraquat exposure (48 hours), found no significant changes to lipid peroxidation as measured by the TBARS assay.

Conversely, AgNP exposure is commonly found to induce lipid peroxidation amongst alternate freshwater species, including zebrafish (Choi *et al,* 2009), medaka (Wu and Zhou, 2012) and mussels (Gangé *et al,* 2013). Making direct comparisons between the aforementioned studies and this investigation is difficult for numerous reasons, many of which may be attributed to discrepancies between experimental conditions and design, e.g. AgNP particle physico-chemical properties, media selection, concentration, dispersion method, and length of exposure. Additionally, rather than using a specific anatomical section of tissue (as in a number of fish and mollusc experiments – liver, gills etc), the entire, homogenised body of *L. variegatus* was used in SOD, CAT and lipid peroxidation assays.

Biological differences between species, namely the effectiveness of antioxidant defence systems must also be taken into consideration. Based on elevated CAT activity and SOD content recorded as part of this investigation, following 24 hours of NM-300K exposure, it is plausible that the antioxidant defence system of *L. variegatus* were capable of resisting the onset of lipid peroxidation. However, future studies investigating protein and DNA damage could be used to assess this theory.

Due to the novelty of this research, it is not possible to make direct comparisons with similar AgNP-*L. variegatus* studies, however, similar conclusions have been drawn for mouse fibroblast cells which, when exposed to AgNP experienced a 1.2-fold increase in the activity of the antioxidant enzyme glutathione and a 1.4 depletion in lipid peroxidation (Arora *et al,* 2009). Conversely, in instances where CAT and other

antioxidant enzyme activity is inhibited during AgNP exposure, as descried for zebrafish by Choi *et al,* (2009), the accumulation of hydrogen peroxide and other oxyradicals can lead to oxidative damage in the form of lipid peroxidation.

Using short-term, aquatic *L. variegatus* exposures (96 hours), Khan *et al* (2015) speculated that AgNP toxicity is directly related to Ag (dissolved and NP) bioavailability and accumulated tissue burdens. In freshwater, Ag can undergo chlorocomplexation – potentially reducing its bioavailability. Although bioavailabilty may be reduced under such circumstances, uptake of Ag (either in dissolved or NP form) is not completelty nullified. In this current study, it is possible that enough Ag (dissolved and NP) was bioavailable and accumulated by *L. variegatus* to stimulate an increase in SOD and CAT activity, however due to chlorocomplexation, it is possible that tissue burdens were not great enough to induce lipid peroxidation.

Although TiO_2NPs exposure has previously demonstrated an increase in MDA for the oligochaete, *E. fetida* (Hu *et al,* 2010), no such increases were observed for *L. variegatus* exposed to NM-104. A lack of lipid peroxidation supports the theory previously hypothesised in section 4.4.1, whereby NM-104 does not elicit oxidative stress in *L. variegatus* and can be considered non-toxic towards this organism.

4.5 Conclusions

A time and concentration-dependent increase in SOD and CAT was evident in *L. variegatus* exposed to NM-300K, indicating the potential for the NP to cause oxidative stress. The results of this investigation suggest that the activity of both enzymes are linked, whilst providing evidence towards the theory that aquatic invertebrates rely upon antioxidant enzymes for protection against ROS. No lipid peroxidation was observed for *L. variegtus* exposed to NM-300K. Whilst these results suggest that CAT and SOD may offset the damaging effects of NM-300K-mediated oxidative stress in *L. variegatus,* it is possible that damage may still have occurred via underlying molecular changes. The response of *L. variegatus* transcriptome and metabolites have been investigated in relation to more traditional contaminants (Agbo *et al,* 2013), however, to this date, remain an unexplored area in relation to NPs. Although not possible within the scope of this investigation, molecular techniques could be used in future research to gain further insight toward *L. variegatus* response to nanoparticle exposure.

Chapter 5: Chronic toxicity testing – investigating the toxicity of NM-300K and NM-104 spiked sediment towards *L. variegatus.*

5.1 Introduction

Sediments will form an important sink for NPs as their popularity and use continue to rise (Koelmans *et al,* 2008). As outlined in detail in section 1.6.3, *L. variegatus* are intimately associated with sediments and are thus extremely susceptible to accumulate potential contaminants through an array of exposure routes (OECD, 2008). Baun *et al* 2008 (figure 5.1) illustrate the numerous direct and indirect routes through which NPs are expected to become associated with aquatic sediments and highlight the significance of sediment dwelling invertebrates within the food web.

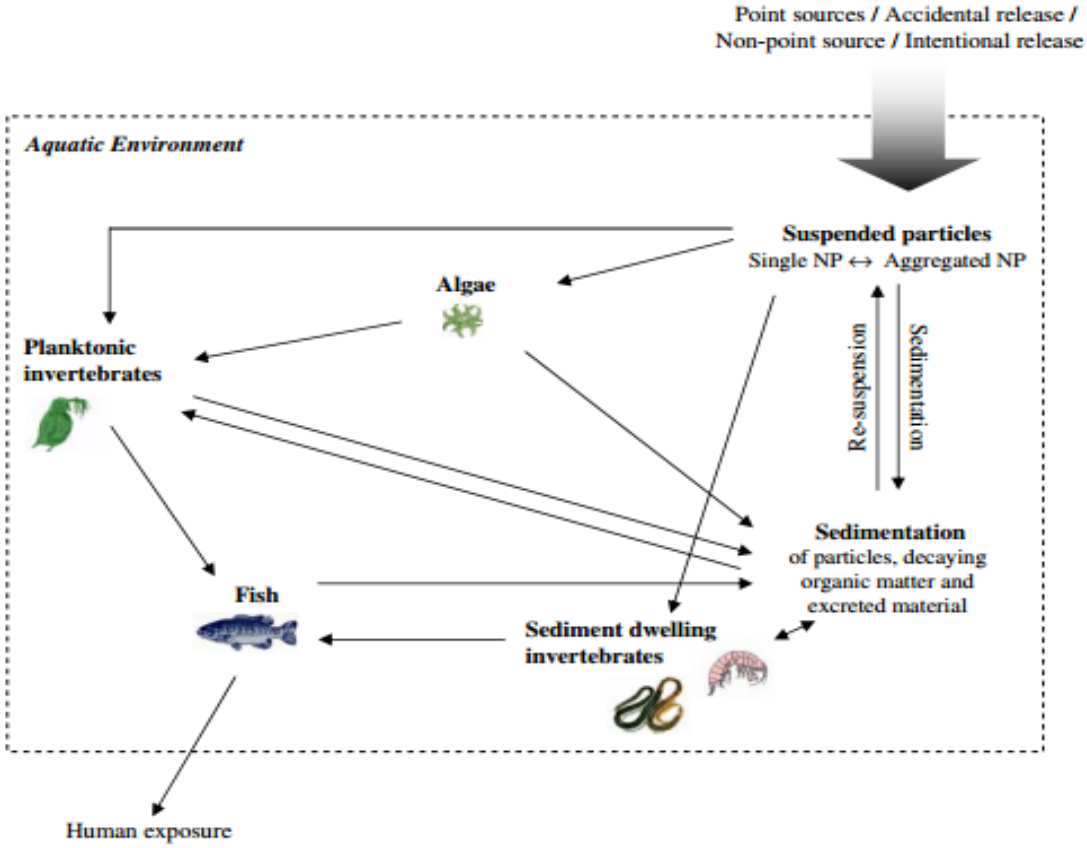

Figure 5.1 The significance of sediment dwelling invertebrates and the possible routes of NP routes of exposure following their release into the aquatic environment (Source: Baun *et al,* 2008).

In addition to the potential bioaccumulation of NPs in higher species such as fish (and eventually humans), detrimental effects upon *L. variegatus* as a result of NP exposure

may initiate changes within the structure and function of freshwater environments. Despite the likelihood of NP-contaminated sediments in freshwater environments, very few publications to date have investigated the toxicity of NP-contaminated sediments towards environmentally significant sediment species.

5.1.1 Freshwater sediments and their contamination

Sediments can be broadly defined as all consolidated and unconsolidated material at the base of aquatic systems, and include a variety of materials with differing physical and chemical properties, including but not limited to, organic matter, silts, clay, sands, rocks and boulders (Palmer *et al*, 2000). Allocthonous sources of sediments include materials that have been geographically transported and subsequently sedimented, including eroded soil, river/lake bed material (predominantly sand and gravel), as well as suspended minerals and organic matter from groundwater and wastewater tributaries (Kalinowski and Załęska-Radziwiłł, 2011). Conversely, autochthonous sources of sediment materials are those which originate from within the aquatic system and may be derived from; the precipitation of organic and inorganic materials such as calcium carbonate, iron and magnesium hydroxides, phosphorous compounds, in addition to the accumulation of dead plant and animal remains on the sediment surface (Kalinowski and Załęska-Radziwiłł, 2011).

Sediments are integral to the function of healthy aquatic systems and provide a habitat for various animal and plant species. Palmer *et al* (2000) define freshwater sediment biota as *"organisms living within aquatic sediments, on aquatic sediments or closely associated with aquatic sediments at some stage of their life"*. Although the quantification of biodiversity is challenging, given that the majority of sediment species are microscopic and live deep within sediment, the global biodiversity of sediment habitats is thought to comprise more than 100,000 benthic species, 10,000 algal species, and more than 20,000 protozoan species (Palmer *et al,* 1997).

Given the ecological value held by freshwater sediment environments and their high susceptibility to contaminants (illustrated in figure 5.1), investigating the behaviour and toxicity of potential contaminants within sediment exposure settings is vital. Sources of sediment contamination can be broadly divided into two categories, point and non-

point (or diffuse). Point sources of contamination such as wastewater effluents are; identifiable, steady in regard to flow and concentration and tend to be independent of meteorological factors (Vink and Behrendt, 2001). Conversely, diffuse sources of contamination are often the consequence of meteorological factors and by definition, highly dynamic and widespread (Vink and Behrendt, 2001). Diffuse sources of sediment contamination, such as; surface runoff, groundwater, sewer overflows and are much more challenging to predict and reduce.

The USA Environmental Protection Agency (EPA) currently cites: nutrients (namely phosphorus and nitrogen compounds), bulk organics such as oil and grease, halogenated hydrocarbons, (e.g. dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs)) and metals (including; lead, cadmium, mercury, iron and magnesium) as the major contaminants of sediments (US EPA, 2005). Such contaminants typically display a high affinity with sediments (particularly the organic fraction) and thus can persist for long periods of time (years or decades), potentially posing a huge risk to benthic organisms. The re-suspension of sediment-associated contaminants, via bioturbation or anthropogenic means such as dredging or fishing (Eggleton and Thomas, 2004) can pose a threat to aquatic biota, in particular filter feeders. Consequently, development of ecotoxicological test methods for aquatic environments have become an area of focus over recent times (Kalinowski and Załęska-Radziwiłł, 2011). Sediment ecotoxicology can be defined as the investigation of potential contaminants that adsorb to fine particles and organisms that live either in the sediment or are impacted by it and which are directly or indirectly exposed to adsorbed contaminants (SedNet, 2002).

5.1.2 NP contamination of sediments

The mobility of NPs in the aquatic environment is dependent on their aggregation/agglomeration and deposition, which, in turn are dependent upon abiotic factors such as pH and ionic strength. Under favourable conditions (i.e. pH near the point of zero charge), NPs will aggregate/agglomerate, predominantly via Brownian diffusion and become less likely to travel extensive distances (i.e. experience sedimentation) (Petosa *et al,* 2010). Considering the well documented uncertainties

surrounding NPs (i.e. fate, behaviour and toxicity in the environment), understanding their toxicity in sediments is of upmost importance to freshwater benthic organisms and the ecosystem in general.

5.1.3 Current sediment protocols for L. variegatus toxicity testing.

Although a number of protocols exist for the hazard and risk characterisation of standard chemicals in the environment, they cannot always be applied to NPs due to their unique physicochemical properties. While current risk assessment protocols may be generally applicable to NPs, a certain amount of caution has to be taken given the gap in NP toxicity knowledge (Linkov *et al,* 2007). The testing of pre-existing protocols and the development of NP-specific environmental risk protocols are seen as a way of instilling public confidence in safe, environmentally responsible products of nanotechnology.

A number of sediment toxicity (ASTM, 2002; EPA, 2000) and bioaccumulation (ASTM, 2000; Phipps *et al*, 1993) protocols exist for the testing of sediment-associated chemicals towards benthic species. Based on such protocols, the Organisation for Economic Co-operation and Development (OECD) has developed a protocol for the testing of chemicals in sediment, specifically designed for use with *L. variegatus.* OECD guideline 225 is designed to assess the effects of prolonged exposure (28 days) of *L. variegatus* to sediment associated chemicals, using reproduction and biomass as endpoints. Within the guidelines, laboratory formulated sediment (artificially prepared in the laboratory) is spiked with a test substance in order to simulate a contaminated sediment.

Although natural sediments offer a more environmentally relevant insight to the toxicity of potential contaminants, they tend to vary in composition according to geographical location and seasonal changes, enhancing the probability of variability and a lack of reproducibility (OECD, 2007). Furthermore, the use of natural sediments may introduce indigenous organisms and/or micropollutants to test conditions, influencing the validity of results. The known composition of laboratory formulated sediments are seen as a way of limiting variability when considering the toxicity of sediment-associated contaminants and are widely used within ecotoxicology. Through the use of formulated

sediments, a standard 'bench mark' can be established, from which it is possible to compare NPs during their screening (Handy *et al,* 2012).

The process of sediment spiking is one of the major issues when applying standard protocols such as OECD 225 to the testing of NPs, due to its influence on bioavailabilty. Two potential methods, each with their own advantages and disadvantages are outlined by Handy *et al* (2012). The first involves the application of NPs in powder form to the dry sediment components, potentially offering a homogenous mix of NPs, whilst the alternative method involves the direct application (or spraying) of NPs in a liquid suspension to the sediment, potentially providing a more environmentally relevant exposure. The lack of standard mixing protocols for sediments spiked with NPs is also highlighted as an issue by Handy *et al* (2012), who suggest a more disciplined and accountable approach to be administered (e.g. a mechanical food mixer with known settings). NPs are often supplied in different forms (i.e. powder or in suspension), meaning certain application methods are not always applicable.

OECD 225 has been successfully used to assess the toxicity of parasiticides (Egeler *et al,*, 2010), fungicides (Seeland *et al,*, 2012), pesticides (Rosa *et al,* 2015), PCBs (Groh *et al,* 2010) and boron (Hall *et al,* 2014), whilst formulated sediment has been used for the testing of NPs in the form of carbon fullerenes (Wang *et al,* 2011; Wang *et al,* 2014). Given the rise of nanotechnology and the importance of freshwater sediments as contaminant sinks, understanding the applicability of current sediment protocols, such as OECD 225, with NPs is of great importance.

5.1.4 Field Flow Fractionation

As outlined in section 2.1.6, ICP-MS is a popular instrument for the measurement of NPs due to its low detection capabilities and wide dynamic range. While ICP-MS provides valuable information in relation to the concentration of NP-associated elements within samples, more information (related to NP characteristics) can be elucidated when coupled to a size-separation technique (Mitrano, 2012).

Within NP research, Field Flow Fractionation (FFF) is a widely approved and versatile suite of elution techniques capable of separating NPs within complex samples (Poda *et*

al, 2011). Among the FFF family, flow FFF is commonly used for environmental applications. Flow FFF separation occurs within a thin ribbon-like channel which is enclosed by one (asymmetrical FFF) or two (symmetrical FFF) porous blocks. Due to the high aspect ratio of the channel, a parabolic flow develops whereby flow velocity increases towards the centre of the flow FFF channel. Following the injection of samples (via the tip flow) and a focusing period, a perpendicular fluid cross flow is applied to the sample flow within the channel. The cross-flow forces particles against the accumulation wall (a semi-permeable membrane) on top of the lower ceramic block (Mitrano, 2012). As NPs build up on the accumulation wall, a counteracting diffuse force is initiated, driving NPs back towards the centre of the channel (Messaud *et al,* 2009). The balance between cross flow and diffuse forces leads to the elution of smaller NPs (occupying high flow velocity zones) before larger particles (occupying low flow velocity zones).

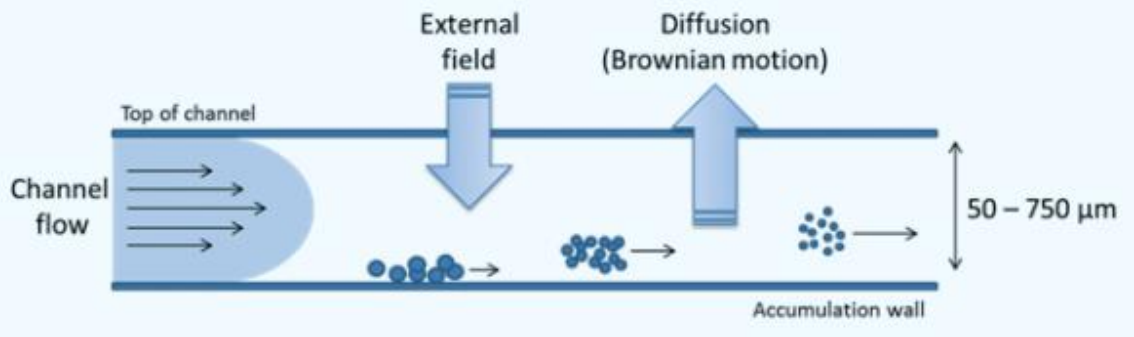

Figure 5.2. Schematic diagram of FFF channel demonstrating the balance between the external field towards the accumulation wall and the diffusion away from this wall (Source: Waegeneers, unpublished)

By combining FFF and ICP-MS, it is possible to detect and analyse NPs (in relation to composition, concentration and size) at low concentrations (ppb) (Mitrano, 2012) within complex media, which is extremely useful within ecotoxicology testing. FFF-ICP-MS has previously been used to detect AgNPs within *L. variegatus* following sub-lethal exposure in sediment (Poda *et al,* 2011, Coleman *et al,* 2013), as well as *D. magna, Pimephales promelas,* and *Pseudokirchneriella subcapitata* (Kennedy *et al,* 2010). Given the potential for *L. variegatus* to accumulate sediment-associated AgNPs, potentially

leading to NP transfer through the trophic levels, understanding uptake is important in relation to risk assessments.

5.1.5 Aims

Through the use of OECD test guideline 225, this research aims to investigate whether current ecotoxicological sediment protocols are applicable to NPs and if so, whether NM-300K and NM-104 contaminated sediments exert toxic effects upon *L. variegatus* in relation to reproduction and dry biomass. Alternate spiking techniques were investigated, as was the role of natural organic matter (NOM) quantity upon toxicity. The suitability of using Field Flow Fractionation (FFF), coupled to Inductively Coupled Plasma Mass Spectroscopy (ICP-MS) analysis, to investigate the uptake and characterisation of NM-300K in sediment exposed *L. variegatus* over time (3, 10, 17 and 28 days of exposure) was assessed.

5.2 Methods

5.2.1 OECD formulated sediment preparation.

All sediment exposures were conducted using OECD formulated sediments (Annex B). The quantity of peat used represents a slight deviation from OECD test guidelines 225, (reduced from 5% to 2%) in accordance with OECD test guidelines 315 (bioaccumulation in sediment-dwelling benthic oligochaetes) in order to closely replicate the low to medium organic content of sediments. Sediment preparation was conducted in accordance with the steps outlined in OECD 225.

Air-dried peat (Westland Garden Health, Irish Moss Peat) was ground to a fine powder using a pestle and mortar and passed through a 0.5 mm metal sieve. Any large plant remains were removed before adding peat powder to Milli-Q water to form a peat slurry (a water volume 11.5 x the dry weight of the peat powder used produced a stirrable slurry). The pH of the resultant slurry was measured and altered to pH 5.5 (±0.5) using $CaCO_3$ and stirred on a magnetic plate stirrer for at least 48 hours at room temperature in order to stabilise pH and establish a microbial component. Following this period, pH was again measured and altered to pH 6 (±0.5). Once prepared, the peat slurry was added to the remaining dry sediment components and Milli-Q water (table 5.1) and mixed briefly by hand using a plastic spatula before being mixed in an Apollo APM10 planetary mixer at 200 rpm. Mixing was performed in two one hour steps to prevent overheating of the mixer. An hour was left between mixing periods, during which the sediment was again briefly mixed by hand. Fresh sediments were made for each exposure

5.2.2 Pilot OECD formulated sediment studies.

In order to establish whether OECD formulated sediment provided a habitat capable of supporting *L. variegatus'* characteristic burrowing behaviour and survival*;* preliminary 14 day exposures were conducted with non-spiked sediments. Previous OECD formulated sediment exposures conducted at HWU by Dr. Shona O'Rourke have recorded the development of anoxic conditions, leading to the death of *L. variegatus* in the absence of contaminants (unpublished observations). In order to investigate

whether an alternate sediment recipe could be used to negate the onset of anoxia, an initial 14-day pilot study was conducted using cellulose in place of peat as a an organic sediment component (anoxic sediment forms due to the microbial decomposition of organic carbon).

Both sediment types were prepared as described in section 5.2.1 (with exception to the peat slurry procedures for the adapted cellulose sediments – where an equivalent portion of cellulose was used). Once prepared, approximately 100 grams (wet weight) of sediment was transferred to Fisherband® 250 ml borosilicate glass beakers using a plastic spatula. OECD medium (200 ml) was carefully added to each beaker to give a 1:4 sediment: overlying water ratio. Beakers were covered with plastic petri dishes and allowed to equilibrate for 48 hours in incubators at 20°C with a photoperiod of 16:8 (light: dark). Following equilibration, 10 healthy, synchronised adult *L. variegatus* of approximately the same size were transferred via wooden pick to each beaker. Five replicates were used for each sediment type.

With worms introduced, beakers were again covered and stored in the conditions previously described - in addition to the supply of a gentle, steady air flow. Worms were closely monitored for sediment avoidance and abnormal burrowing behaviours within the first 48 hours of the experiments. Additionally, sediment surfaces were observed for the presence of faecal pellets, and the pH, temperature and dissolved oxygen content of the overlying water were measured weekly. Any losses of overlying water, (i.e. due to evaporation) were compensated with the careful addition of fresh OECD medium in order to maintain the original sediment: water ratio in test vessels. After 14 days, worms were removed from test vessels by firstly placing beakers on hot plates (at 50-60°C) to initiate worm migration from the sediment to the water column, following which, worms were carefully extracted via Pasteur pipette to petri dishes containing OECD medium. The successful body reversal responses of worms were then assessed as described in section 3.2.9.

5.2.3 Sediment moisture, solid and organic matter content

NP exposures were spiked on a dry-sediment basis, making the determination of OECD formulated sediment moisture and solid content necessary. The organic matter content

was also determined in conjunction with organic matter studies (section 5.2.5). Approximately 25g of formulated sediment (prepared as described in section 5.2.1, termed moist sediment) was added to pre-weighed ceramic weighing boats (n=5), which were weighed again and placed in an oven at 105°C until dry (termed dry sediment). After cooling in a desiccator, crucibles were weighed, allowing the determination of sediment moisture content as a percentage of the dry sediment weight as follows:

$$Moisture\ content\ (\%) = \frac{W_2 - W_3}{W_3 - W_1} \times 100$$

Where:

W_1 = weight of crucible (g).

W_2 = weight of moist sediment and crucible (g).

W_3 = weight of dried sediment and crucible (g).

Crucibles were then heated at 550°C in a muffle furnace for four hours, after which they were carefully removed using tongs, allowed to cool to ambient temperature in a desiccator and weighed again. Sediment organic matter was determined as follows:

$$Organic\ matter\ (\%) = \frac{W_2 - W_3}{W_2 - W_1} \times 100$$

Where:

W_1 = weight of crucible (g).

W_2 = crucible and dry sediment (g).

W_3 = crucible and sediment ash (post muffle furnace) (g).

5.2.4 NP-spiked sediment exposures

Following the determination of sediment in which *L. variegatus* survival and behaviour was deemed acceptable (OECD formulated sediment with 2% peat); range-finding NP-spiked sediment exposures were conducted. Concentration ranges for NM-300K and NM-104 during range-finding testing were, 0, 16.66, 33.33, 66.66, and 133.33 mg/kg dry sediment and 0, 83.33, 166.66, 333.33, 500 and 666.66 mg/kg dry sediment weight, respectively and selected to reflect the greater anticipated toxicity of AgNP in comparison to TiO_2, in accordance with both the results from chapter 3 and the general consensus within the literature. To 250 ml beakers, 86 g of sediment (prepared as described in section 5.2.1) were added in triplicate to prepare each concentration. OECD 225 recommend that 43g dry sediment should be used per 10 *L. variegatus* in order to maintain a dry biomass: total organic carbon (TOC) ratio of no more than 1:50. The above described pilot study was conducted prior to sediment analysis outlined in section 5.2.3 and assumed a solid sediment content of 50% (as opposed to the measured value of 75%). The concentrations of NM-300K and NM-104 described thus represent corrected values.

NPs were applied directly to the moist sediment surface of each test vessel in 2 ml aqueous suspensions via pipette (desired concentration calculated in relation to dry sediment weight). Beakers were subsequently placed in a shaker (Gallenkamp Environmental Shaker Model 10X 400) at 200 rpm overnight to achieve a homogenous mixture of NPs within sediment. This application represented a deviation from the OECD 225 protocol which recommends the spiking of an isolated, dry sand portion of sediment (10g) with 2 ml of the desired test substance, which is then allowed to dry before being incorporated to the remaining sediment which is subsequently mixed. An alternate method was employed from both a practical standpoint, i.e. the spiked sand method requires each sediment-exposure concentration to be mixed individually - a time consuming approach given the availability of only one mixing device, and also to limit NP adhesion to mixing equipment, which would lead to contamination.

Once NPs had been applied and mixed to sediments, OECD water and *L. variegatus,* (following a 48-hour equilibration period) were added to beakers and stored as described in section 5.2.2 for 28 days. Worms were again monitored closely within the first 48 hours of exposure, whilst the water parameters (of one randomly selected vessel

per treatment) described in section 5.2.2 were recorded on a weekly basis (Annex C). Following 28 days of exposure, worms were removed from each beaker as previously described (section 5.2.2) and counted to determine survival and reproduction. Living worms were determined as any of the following:

- Large complete worms without regenerated body regions.
- Whole worms with re-generated segments (either lighter coloured posterior or anterior).
- Newly fragmented, incomplete worms.

Once counted, worms were placed in pre-weighed plastic weighing boats, and killed using one drop of 70% ethanol per weighing boat. Weighing boats were placed in an oven at 100°C (±5°C) overnight, after which their weight was recorded, allowing a determination of worm dry biomass per replicate

Based on the results of the range-finding study, further toxicity tests were performed for NM-300K and NM-104. Once sediment solid content had been determined, the quantity of sediment used within tests was amended to 60 grams per beaker to give approximately 43 grams' dry weight (sediments recorded a ~75% solid content). A concentration range of; 0 (control), 83.33, 166.66, 333.33, 666.66 and 1333.33 mg/dry weight sediment was selected for both NM-300K and NM-104 (3 replicates, n=10) based on range-finding experiments. Worm number and dry biomass were again recorded, and where applicable, dose response curves (as a reduction of worm numbers in relation to the control) were produced using the following equation:

$$100 - \frac{no.\,of\ L.\,variegatus\ in\ exposure}{no.\,of\ L.\,variegatus\ in\ control} \times 100$$

Behavioural responses (body reversal and helical swimming) were also recorded for control worms and worms exposed to sub-lethal (166.66 mg/kg dry sediment)

concentration of NM-300K and NM-104. Behavioural responses were recorded for 10 adult worms, selected randomly from each replicate and assessed in accordance with section 3.2.9.

Exposures were also conducted for the NM-300K dispersant, NM-300K DIS using volumes equivalent to NM-300K concentrations of 0, 333.33, 666.66 and 1333.33 mg/kg dry sediment to determine whether it accounted for any potential NM-300K toxicity. All test conditions remained constant with those employed in the range-finding studies.

5.2.5 Influence of sediment NOM content on NP toxicity

Sediment toxicity tests were performed using alternative quantities of peat to determine whether natural organic matter (NOM) influenced the toxicity of NM-300K in sediment exposures. Tests were conducted as described in section 5.2.4 with peat quantities of 2, 5 or 10 % of the overall dry sediment composition. Sediments were spiked with 666.66 mg/kg NM-300K (3 replicates, n=10), a concentration found to have an effect in previous toxicity tests without evoking 100% mortality. Control samples (3 replicates, n=10), containing the corresponding quantity of peat but without NM-300K spiking were also run in parallel. No toxic concentrations were observed during NM-104 exposures and thus no NOM tests were performed with this particle. NOM contents of sediments were quantified as described in section 5.2.3.

5.2.6 Alternate NP exposure route

The introduction of NM-300K via the water column was investigated as an alternate, potentially more environmentally relevant exposure route for sediment toxicity testing. Non-spiked sediments, prepared as previously described (section 5.2.1) were added (86g) to 250 ml glass beakers, following which 190 ml of OECD medium was added. Beakers were left to equilibrate for 48 hours (20^{o}C, photoperiod 16:8, light: dark) before the addition of 10 worms per beaker. A period of 24 hours was allowed for worms to acclimatise to sediment and exhibit standard burrowing behaviour before an additional

10 ml of OECD medium containing differing concentrations of NM-300K (depending on the desired final concentration) was carefully introduced, with minimal sediment disturbance. A concentration range of 0 (control), 0.5, 2.5, 5, 7.5 and 10 mg/L was employed (3 replicates) for 28 days after which worm numbers and dry biomass were assessed as described in section 5.2.4.

5.2.7 L. variegatus exposure and digestion for FFF-ICP-MS analysis

L. variegatus sediment exposures (3, 10, 17 and 28 days) were conducted at Heriot-Watt University, while all FFF-ICP-MS analysis was conducted at Colorado School of Mines (CSM), USA. OECD sediment was made as described in section 5.2.1, with 250g added to 1 litre beakers (larger exposures were set up as more *L. variegatus* tissue was required). Each beaker (4 per time point) was then spiked with NM-300K (as described in section 5.2.4) to give a nominal concentration of 100 mg/kg dry sediment. Following the addition of OECD medium (1:4 sediment to water ratio) and a 48-hour equilibration period (20^{o}C, photoperiod 16:8 (light: dark)), 25 healthy adult *L. variegatus* of approximately the same size were added to each test beaker. Following exposure after 3, 10, 17 and 28 days (in conditions described in section 5.2.4), worms were removed, rinsed with MilliQ water and depurated in MilliQ water for 6 hours, after which they were digested in 20% tetramethylammonium hydroxide (TMAH) for 24 hours, the first of which was aided in a bath sonicator (Kerry PUL325).

5.2.8 FFF-ICP-MS analysis

An AF2000 mid temperature (MD) Asymmetrical Flow Field Flow Fractionation (AF4) instrument (Postnova Analytics, Salt Lake City, UT, USA) coupled with a PerkinElmer NexION 300D ICP-MS was used throughout the investigation. FFF parameters used within this investigation (listed in table 5.1) remained consistent throughout with the exception carrier fluids concentrations which were altered in an attempt to investigate their influence on the sensitivity of the readings.

Table 5.1. FFF parameters

Membrane type	Regenerated cellulose
Membrane porosity	10 kDalton
Spacer width	350 µm
Sample injection volume	100 µL
Detector flow	1 ml/min
Cross flow	1.20 ml/min
Flush time	5 mins
Carrier fluid	0.05% NaN_3 0.025%FL-70 surfactant (Fisher Scientific) *

* Concentration of carrier fluids were diluted by a factor of 2.5 (in MilliQ water) during some FFF-ICP-MS runs.

TMHA-digested *L. variegatus* tissues were diluted (1:20) with MilliQ water before analysis. A volume of 30 µL was then manually injected into the PN5120 injector bracket (Postnova Analytics, Salt Lake City, UT, USA). Two PN1130 isocratic pumps (tip and focus) were used to create the AF4 separation field conditions, while channel flow conditions facilitated the direct connection of FFF effluent with the ICP-MS nebulizer. One silver isotope (Ag^{107}) was measured for NM-300K samples, while a gold isotope (Au^{197}) was also measured for samples including an internal AuNP (30 nm, NanoComposix) standard. Internal AuNP standards (0.05 mg/L) were added to *L. variegatus* samples as a means of monitoring instrument sensitivity (with AuNP recording a different elution time to that of NM-300K). Each FFF-ICP-MS was programmed to start with a 10-minute focusing period followed by a 14 minute elution time (NM-300K elution found to be within this time period when suspended in MilliQ water) and a rinse step (5 mins) before the next sample was introduced.

5.2.9 Statistical analysis

Data were assessed for normality using the Shapiro-Wilk test, while statistical differences were assessed using one-way ANOVA analysis and least square difference (LSD) tests (IBM SPSS statistics 22). Concentration-response curves (four-parameter logistic) were fitted in GraphPad Prism® 6. Response values were normalised and plotted against the logarithm of NM-300K concentration, from which an LC_{50} value was generated.

5.3 Results

5.3.1 Pilot sediment-only tests

Results from pilot sediment only experiments concluded that formulated OECD sediment, with peat as a source of natural organic matter, as opposed to cellulose was considerably more favourable for *L. variegatus* health and survival. The vast majority of worms in peat sediments buried head first into the sediment (as displayed in figure 5.3a) within the first 30 minutes of their introduction and remained there for the duration of the test period (14 days). Conversely, a high proportion of worms in cellulose adapted sediments remained on the sediment surface within the first 48 hours and continued to display sediment avoidance (figure 5.3b) throughout the test period. Incidents of faecal pellets were also observed for cellulose adapted sediments, whilst none were recorded for peat sediments.

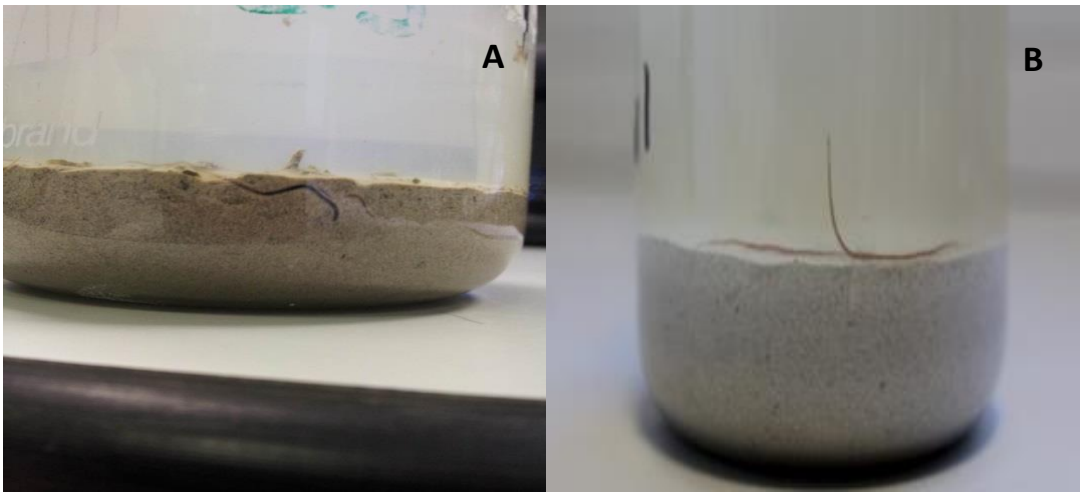

Figure 5.3 *L. variegatus* interaction with OECD formulated sediment with peat (A) and cellulose (B) as the organic matter fraction.

Significantly poorer behavioural responses ($p<0.001$) were observed for worms exposed in cellulose-containing sediments in comparison with peat-containing sediments, further underlining its unsuitability for toxicity testing (figure 5.4). Consequently, OECD formulated sediment with a peat content of 2% were used within NP-spiked sediment toxicity testing.

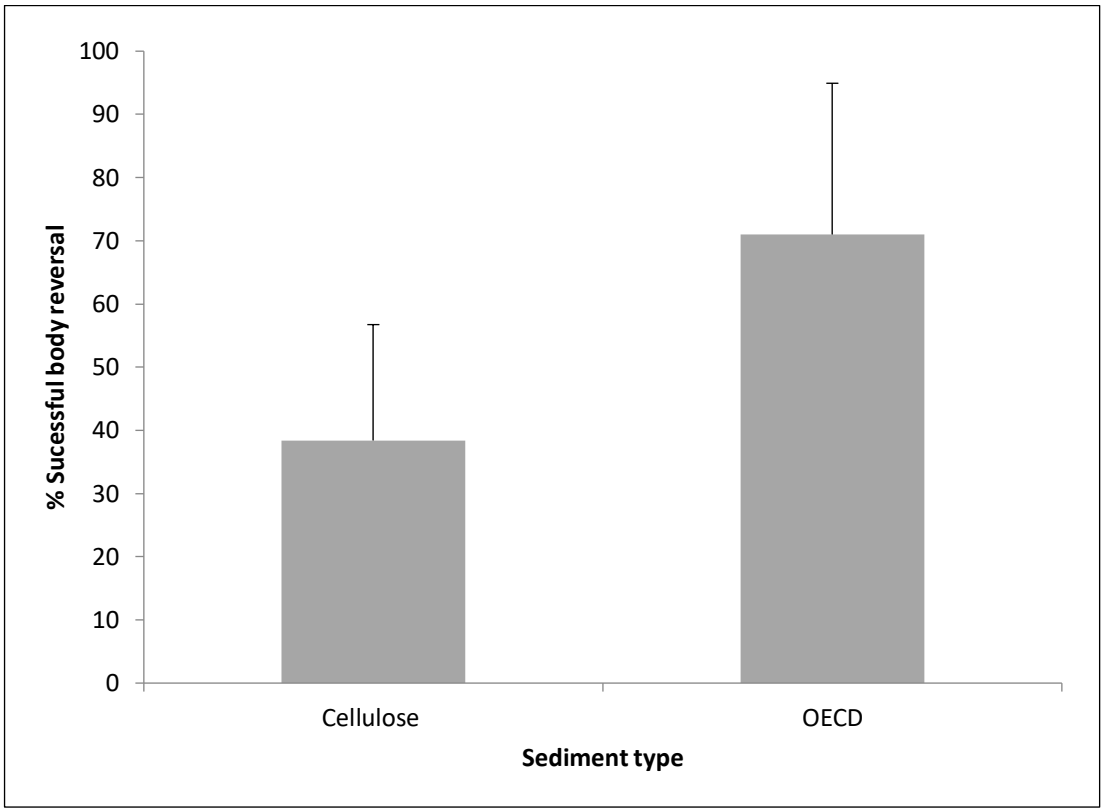

Figure 5.4 Average successful body reversal behaviour of *L. variegatus* inhabiting peat and cellulose OECD formulated sediments at 14 days (data expressed as average +SD, n=5).

5.3.2 Range-finding 28-day sediment exposures

Initial NP-spiked sediment toxicity tests revealed low toxicity in relation to both average dry worm biomass and total numbers recovered following 28 days of exposure (figures 5.5a and b respectively) for both NM-300K and NM-104, concentrations ranging from 0-133.33 and 0- 666.66 mg/kg, respectively. No significant differences in either biomass or worm numbers in either NM-300K or NM-104 exposures compared to the control group ($p>0.05$).

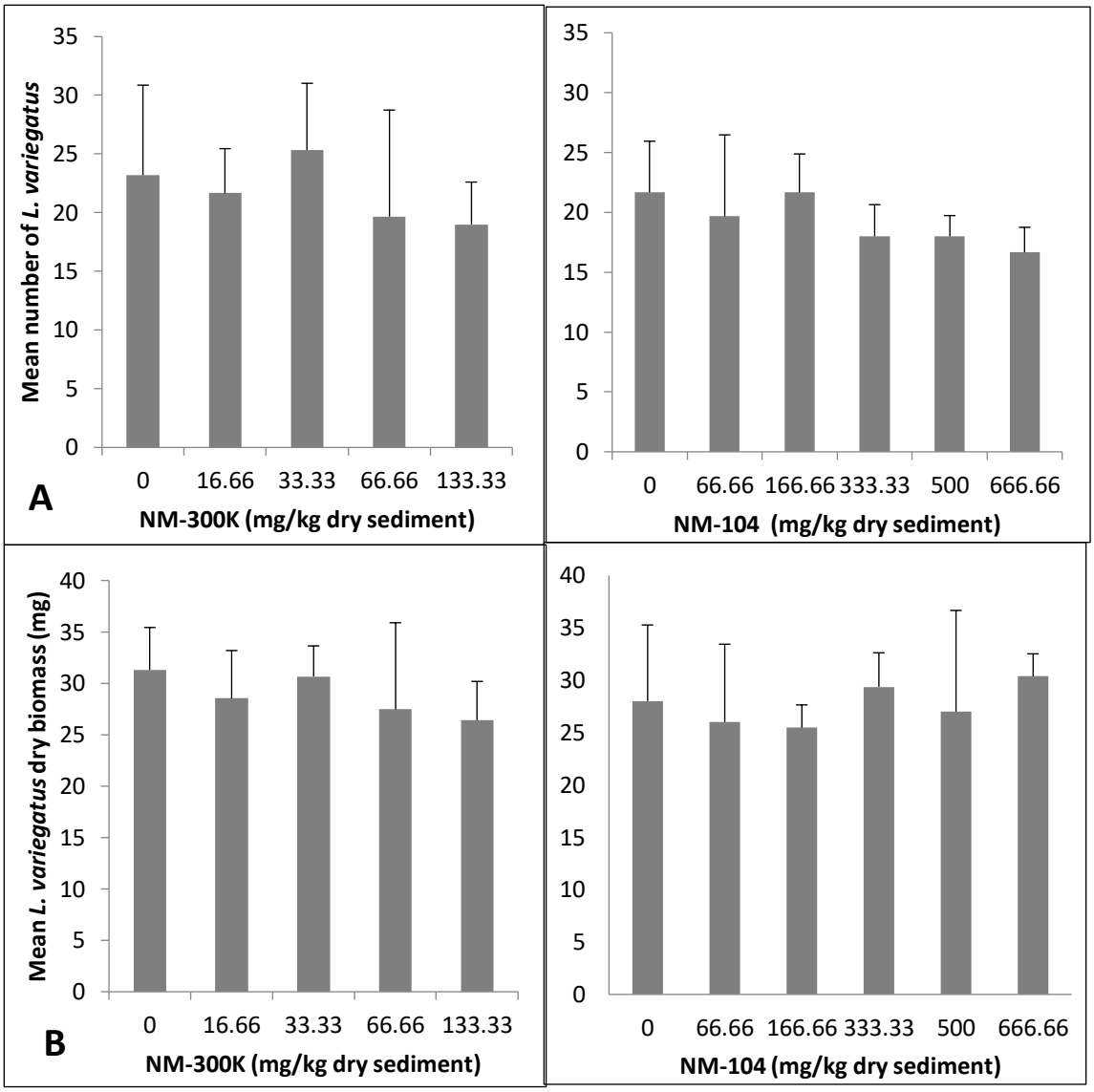

Figure 5.5 Average *L. variegatus* numbers (A) and dry biomass (B) following exposure to NM-300K and NM-104 spiked sediments for 28 days (+SD, n=3).

5.3.3 Further NM-300K spiked sediment exposures

Based on the results in section 5.3.2, further 28-day spiked sediment exposures were conducted to ascertain whether any toxic effects would be observed using an extended NM-300K concentration range (0-1333.33 mg/kg dry sediment). 100% mortality was recorded for worms exposed to a concentration of 1333.33 mg/kg dry sediment (figure 5.6) (significantly different from all over samples, $p<0.05$). A concentration of 666.66 mg/kg resulted in an almost 50% decrease in worms (in comparison to controls and found to be significantly different; $p<0.05$) (Figure 5.6). Worms recovered from 666.66 mg/Kg exposures were predominantly fragmented or whole with signs of regeneration.

An LC_{50} value of 671.2 mg/kg for NM-300K was recorded. Given the absence of worms in 1333.33 mg/kg dry sediment samples, no biomass could be recorded (figure 5.7). The biomass of 666.66 mg/kg sediment worms was the only sample found to be significantly reduced in comparison to the control ($p<0.05$).

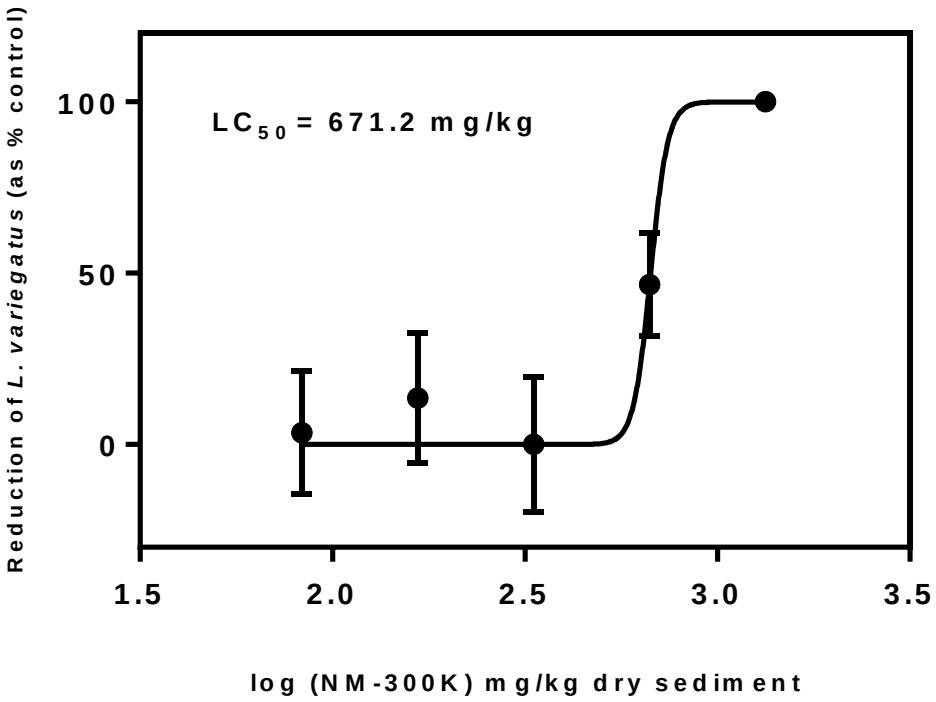

Figure 5.6 Concentration-response curve displaying mean loss of *L. variegatus* (compared to controls) following exposure to NM-300K-spiked sediments (83.33 – 133.33 mg/kg dry sediment) for 28 days (±SD, n=3).

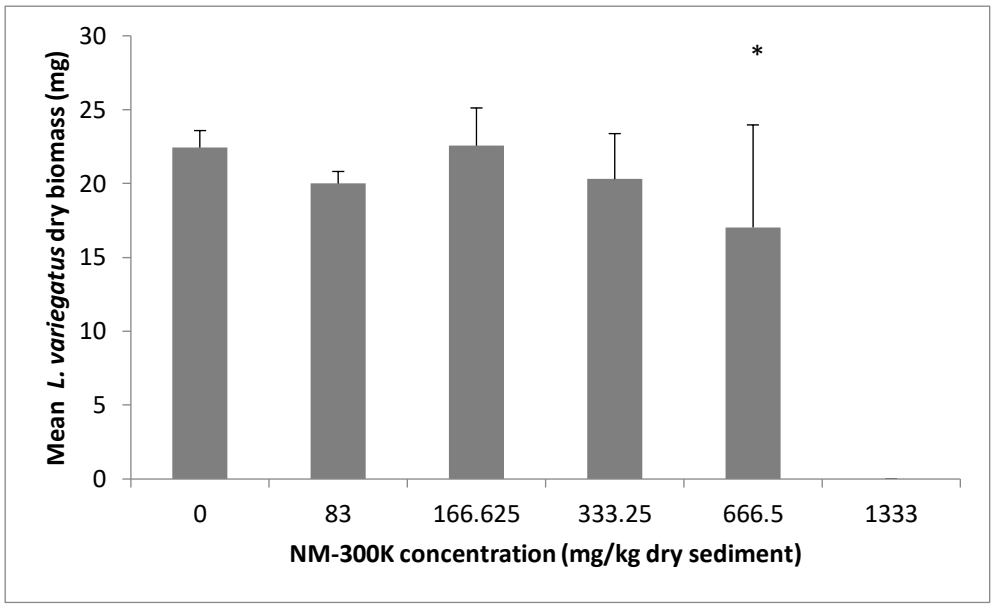

Figure 5.7 Average *L. variegatus* biomass following exposure to NM-300K-spiked sediments for 28 days (+SD, n=3). * denotes significant difference from the control ($p<0.05$).

5.3.4 NM-300K DIS spiked sediment exposures.

Despite a slight decline in *L. variegatus* numbers at a concentration equivalent to 1333.33 mg/kg dry sediment, no significant differences were detected between samples (figure 5.8a). The dry biomass of *L. variegatus* exposed to 333.33 and 666.66 mg/kg dry sediment was found to significantly ($p<0.05$) increase in comparison to control samples. Despite a significantly lower recording than both other NM-300K DIS spiked sediments, worms exposed to 1333.33 mg/kg dry sediment were found to have a biomass similar to those in control exposures ($p>0.05$). These results suggest that NM-300K DIS holds no toxic influence upon *L. variegatus* in sediment exposures and thus any toxicity observed during NM-300K exposure is a direct result of NM-300K alone.

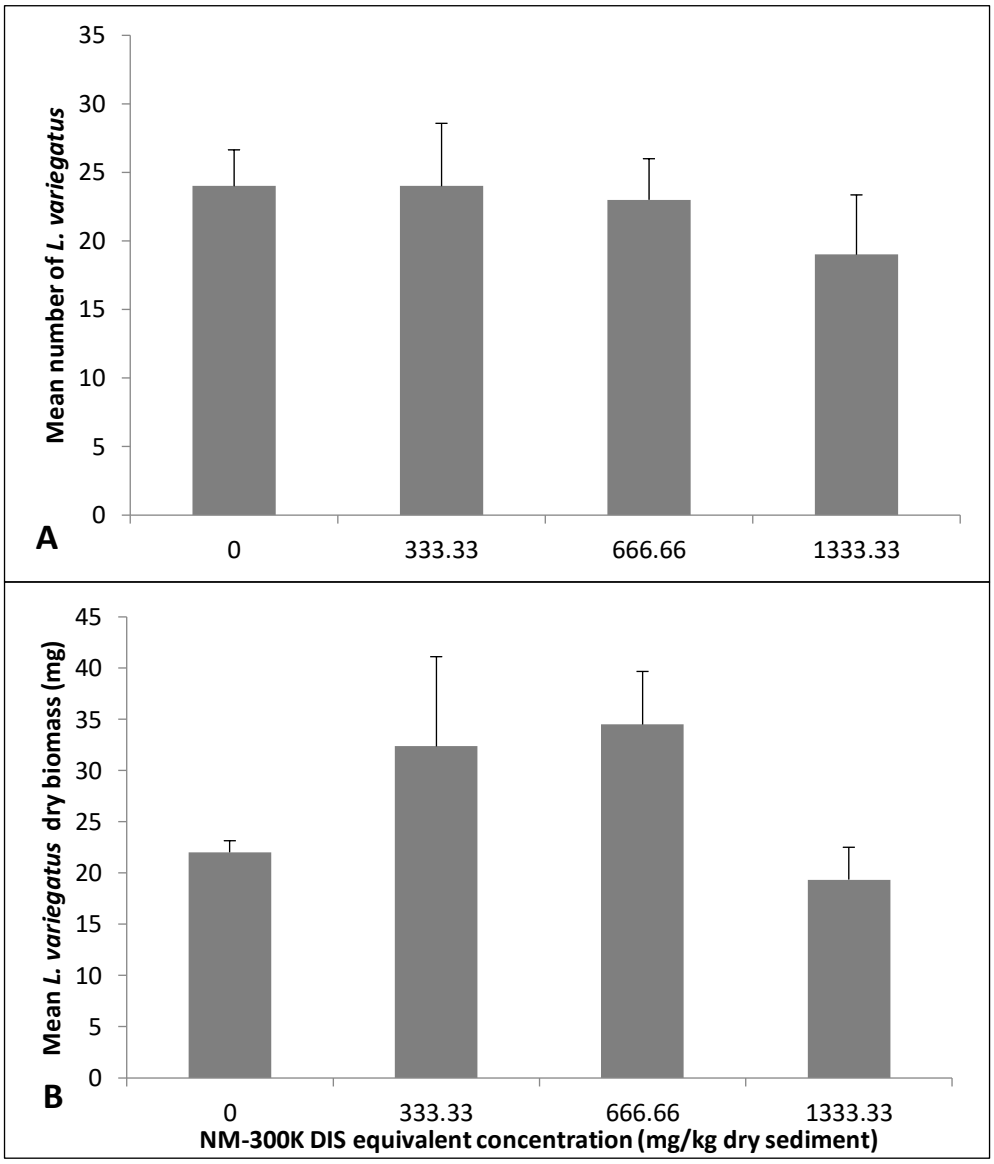

Figure 5.8 Average *L. variegatus* numbers (A) and dry biomass (B) following exposure to NM-300K-spiked sediments for 28 days (+SD, n=3). * denotes a significant ($p<0.05$) difference from control and 1333.33 mg/kg dry sediment samples.

5.3.5 Further NM-104 spiked sediment exposures

Significant ($p<0.05$) decreases in worm numbers were recorded in 1333.33 mg/kg dry sediment exposures (Figure 5.9a), albeit without evoking 100% as recorded for NM-300K. Worms recovered from 1333.33 mg/kg were predominantly fragmented or whole with signs of regeneration. No other test concentrations stimulated a decline in *L. variegatus* numbers (figure 5.9a). Despite an evident decline in dry biomass, no significant differences were recorded between 133.33 mg/kg dry samples and controls. A NM-104 concentration of 83.33 mg/kg was found to significantly increase worm biomass in relation to control (figure 5.9b).

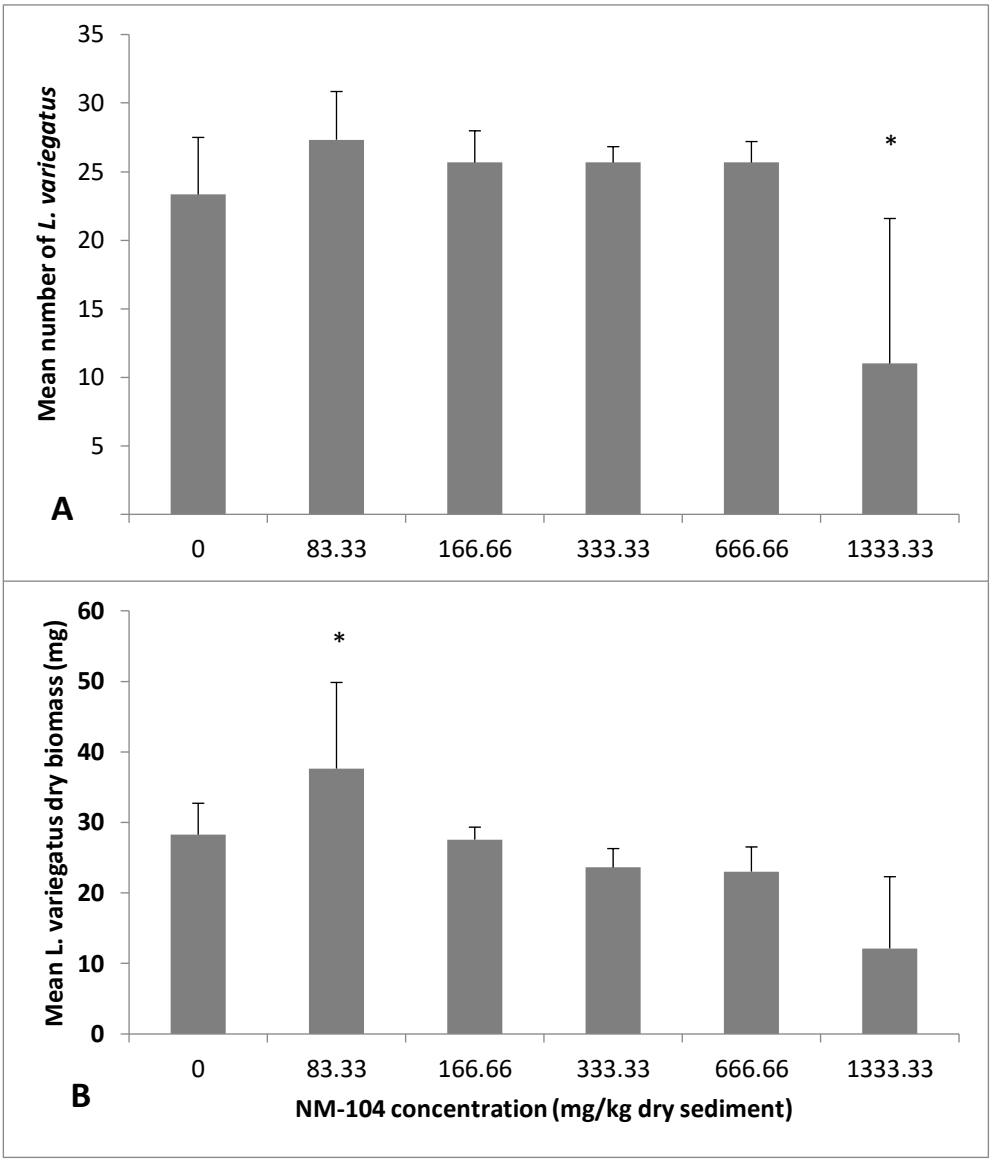

Figure 5.9 Average *L. variegatus* numbers (A) and dry biomass following exposure to NM-104 spiked sediment for 28 days (+SD). * denotes a significant difference ($p<0.05$) from control samples.

5.3.6 Behavioural responses

Although *L. variegatus* body reversal responses proved to be greater than helical swimming responses (figure 5.10), in control, NM-300K and NM-104 sediment exposures, no significant differences were detected between the two ($p>0.05$). Based on both body reversal and helical swimming responses treatments could be ranked control>NM-300K>NM-104, however similarly, no significant differences were recorded between the three sediment types ($p>0.05$).

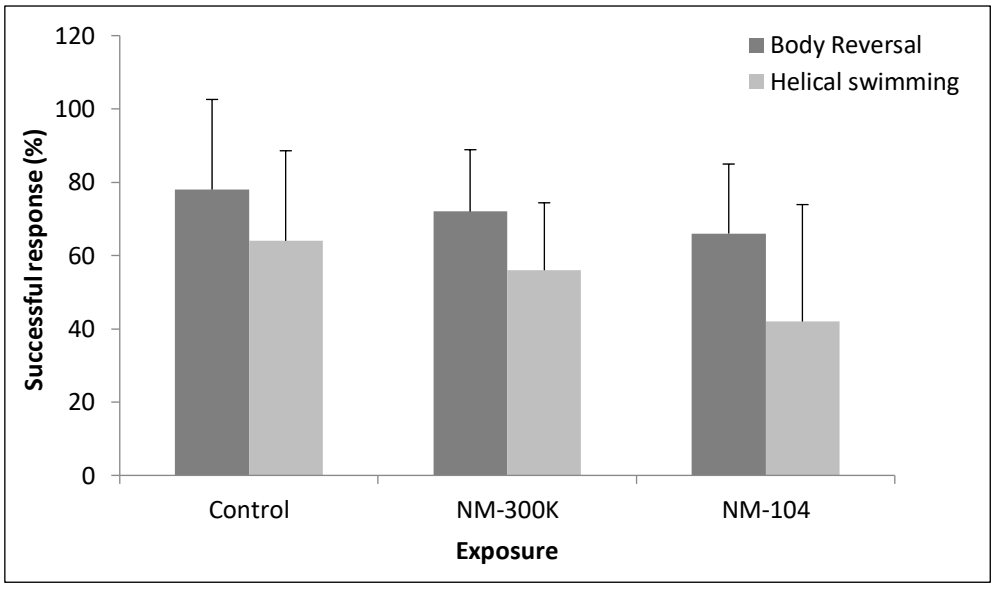

Figure 5.10 Average body reversal and helical swimming responses of *L. variegatus* exposed to control and NM-300K and NM-104 spiked sediments (166.66 mg/kg dry sediment) for 28 days (+SD).

5.3.7 Influence of sediment NOM content on NM-300K toxicity

As anticipated, both *L. variegatus* numbers and biomass were significantly greater in sediments with no NPs, whilst a NOM dependent decrease in toxicity was evident (figures 5.11a and b). However, the number of *L. variegatus* recovered from both spiked and non-spiked sediments was poor, thus influencing the validity of results. Control sediments (using 2% peat component) in previous toxicity tests recovered in the region of 20-25 worms, approximately double the number recorded in 2% peat sediments (11.3) as part of this investigation. Furthermore, 666.66 mg/kg dry sediment, a NM-300K concentration previously found to reduce worm numbers by almost 50% in studies

using 2% peat sediments, but caused 100% mortality when NOM was included in the sediment. Whilst worm survival was recorded in 5 and 10% peat-content sediments spiked with NM-300K; indicating a decrease in toxicity with an increase in NOM content, the number of worms recovered from the corresponding control sediments were similarly lower than anticipated. Worm biomass, as a direct result of reduced worm numbers was also abnormally low (figure 5.9b), particularly within 2% peat-content sediments.

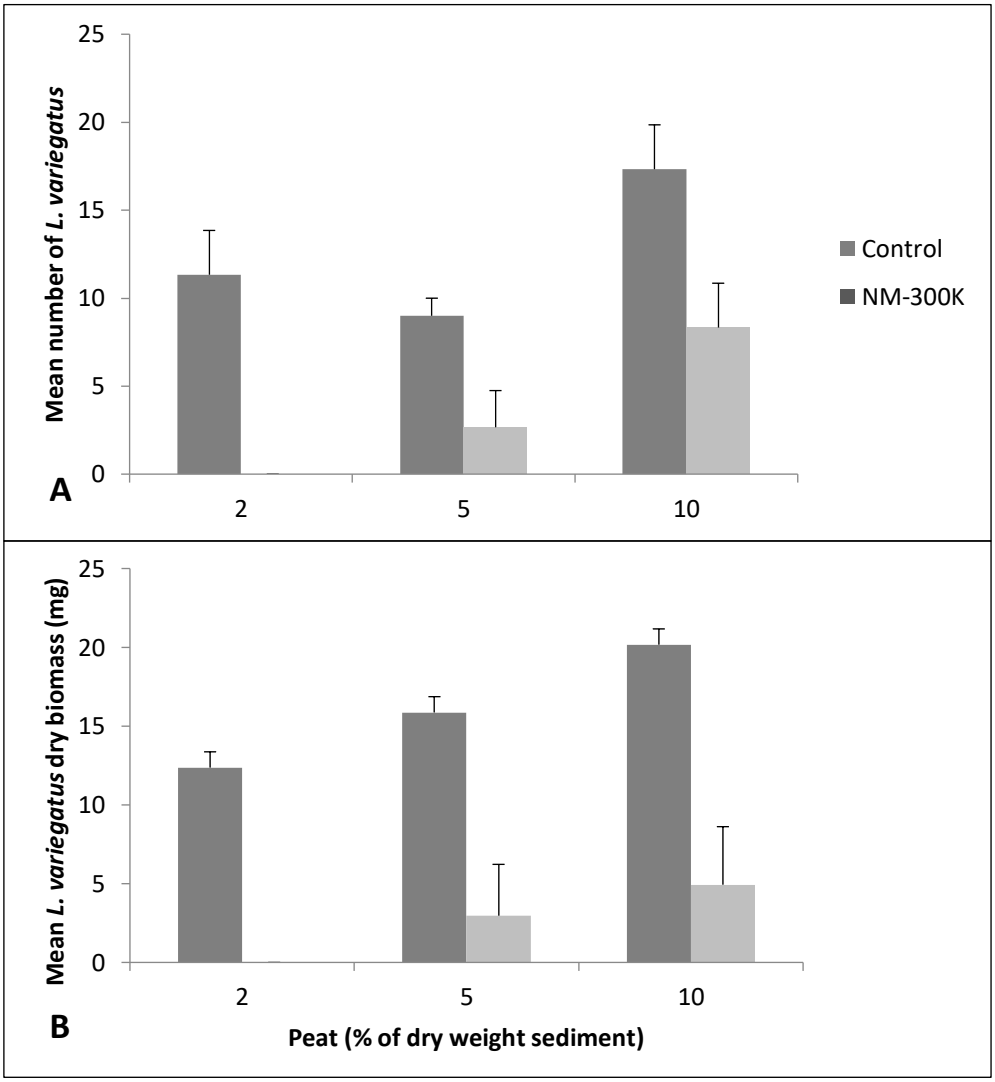

Figure 5.11 Mean number (A) and dry biomass (B) of *L. variegatus* in sediment containing 2, 5 or 10% peat with and without NM-300K spiking (+SD).

5.3.8 Alternate NP exposure route

A decline in worm numbers with an increase in NM-300K concentration within the overlying water column was observed for alternate exposure experiments (table 5.2). However, a >1.8 factor increase in worm numbers was not recorded for control groups, indicating poor worm health and a failure of OECD 225 test requirements. Consequently, the validity of results was compromised and NM-300K cannot be confirmed for the mortality observed.

Table 5.2 Mean number of *L. variegatus* and successful body reversal behaviour of recovered worms following 28-day sediment exposure with NM-300K-spiked overlying water (±SD).

Concentration mg/L	Mean number of *L. variegatus* (±SD)	Mean successful body reversal (%)
0	9.33 (±0.57)	72.22 (±5.09)
0.5	9 (±4.58)	74.44 (±8.38)
2.5	11 (1.73)	75.55 (±10.71)
5	1.66 (±2.88)	22.22 (±38.49)
7.5	1.66 (±2.88)	60
10	3.33 (±5.77)	70

5.3.9 FFF-ICP-MS

FFF-ICP-MS conducted with NM-300K (0.1, 0.5 and 1 mg/L) in MilliQ water (no *L. variegatus* tissue) confirmed a retention time of approximately 470 seconds (figure 5.12) (FFF-ICP-MS retention time is related to NP size, ICP-MS response is related to concentration). A concentration dependent relationship was evident between NM-300K concentration and the ICP-MS response recorded – whereby 1 mg/L recorded the highest response, ~720000 cps (counts per second) and 0.1 mg/L NM-300K recorded the lowest (~60000 cps). Void peaks (after ~60 seconds) are formed by small complexes of dissolved Ag with surfactant molecules while peaks recorded after ~900 seconds were initiated by the rinsing of the FFF (figure 5.12).

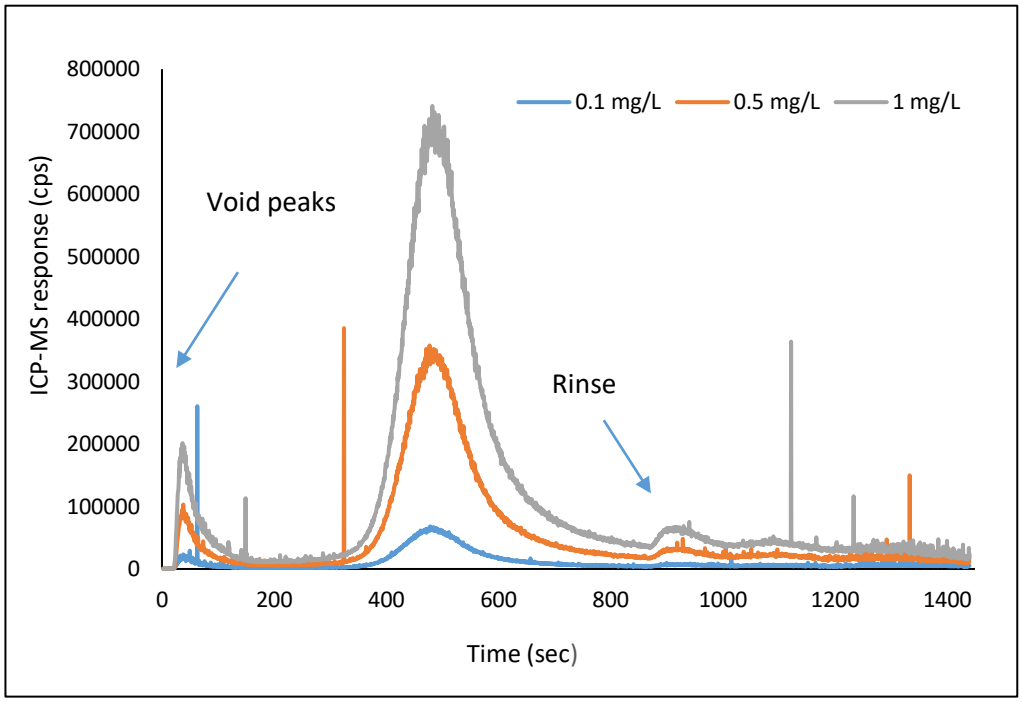

Figure 5.12. FFF-ICP-MS fractograms of NM-300K (0.1, 0.5, 1 mg/L) suspended in MilliQ water, n=1.

Following initial testing of digested *L. variegatus* tissue exposed to 100mg/kg dry sediment NM-300K, Ag peaks (with a retention time similar to that of NM-300K in MilliQ water, figure 5.12) were only detected in five samples across the 3,10,18 and 28 day samples (figure 5.13). From these samples, 18-day *L. variegatus* tissue recorded the lowest reading, with 3 and 28 day tissue recording marginally higher responses, followed by 10 day tissue which recorded a notably higher Ag concentration – suggesting that worms contained NM-300K.

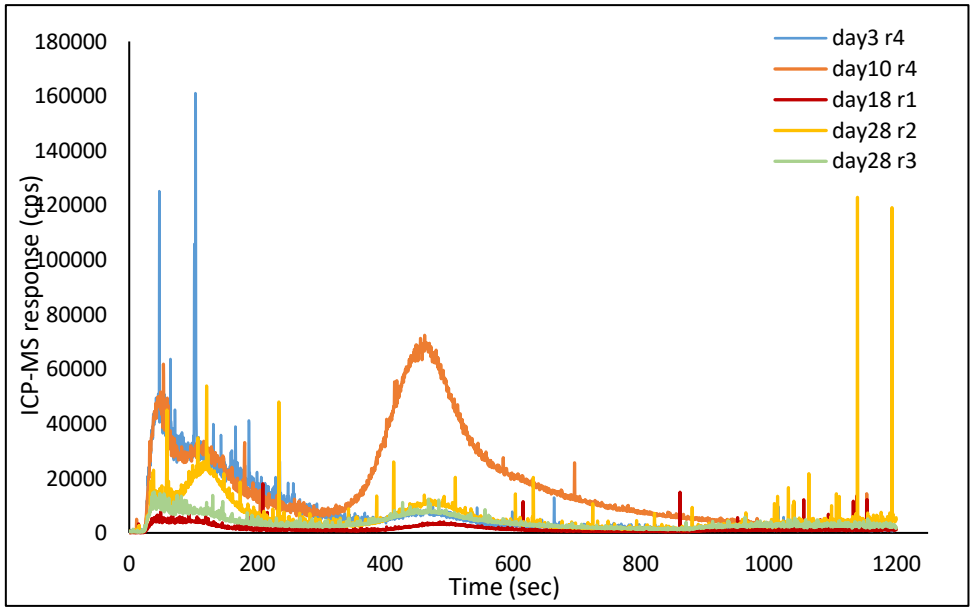

Figure 5.13. FFF-ICP-MS factograms for NM-300K in *L. variegatus* exposed to 100 mg/kg d.w. NM-300K-spiked sediments for 3, 10, 18 and 28 days, r= replicate, n=1.

To determine whether *L. variegatus* tissue samples which recorded no Ag peaks in was due to a lack of NM-300K uptake by *L. variegatus* or related to FFF-ICP-MS methodology, two samples previously shown to contain NM-300K (28 day samples, replicate 2 and 3 - displayed in figure 5.12) were processed again. Both 28 day samples (replicates 2 and 3) originally recorded ICP-MS peaks of approximately 10,000 and 8,000 counts per second after approximately 470 seconds, respectively, however after processing numerous samples (in one day), a loss in ICP-MS sensitivity was evident in both samples (figure 5.14a and b). No ICP-MS peak was recorded after 470 seconds in replicate 2 (approximately 500 counts per second) (figure 5.13a) while after the same time period in replicate 3, an ICP-MS peak of approximately 25,000 counts per second was recorded – a value over two thirds lower than the original peak (figure 5.13b). These results indicate that after the processing of *L. variegatus* samples, a loss in ICP-MS sensitivity was experienced.

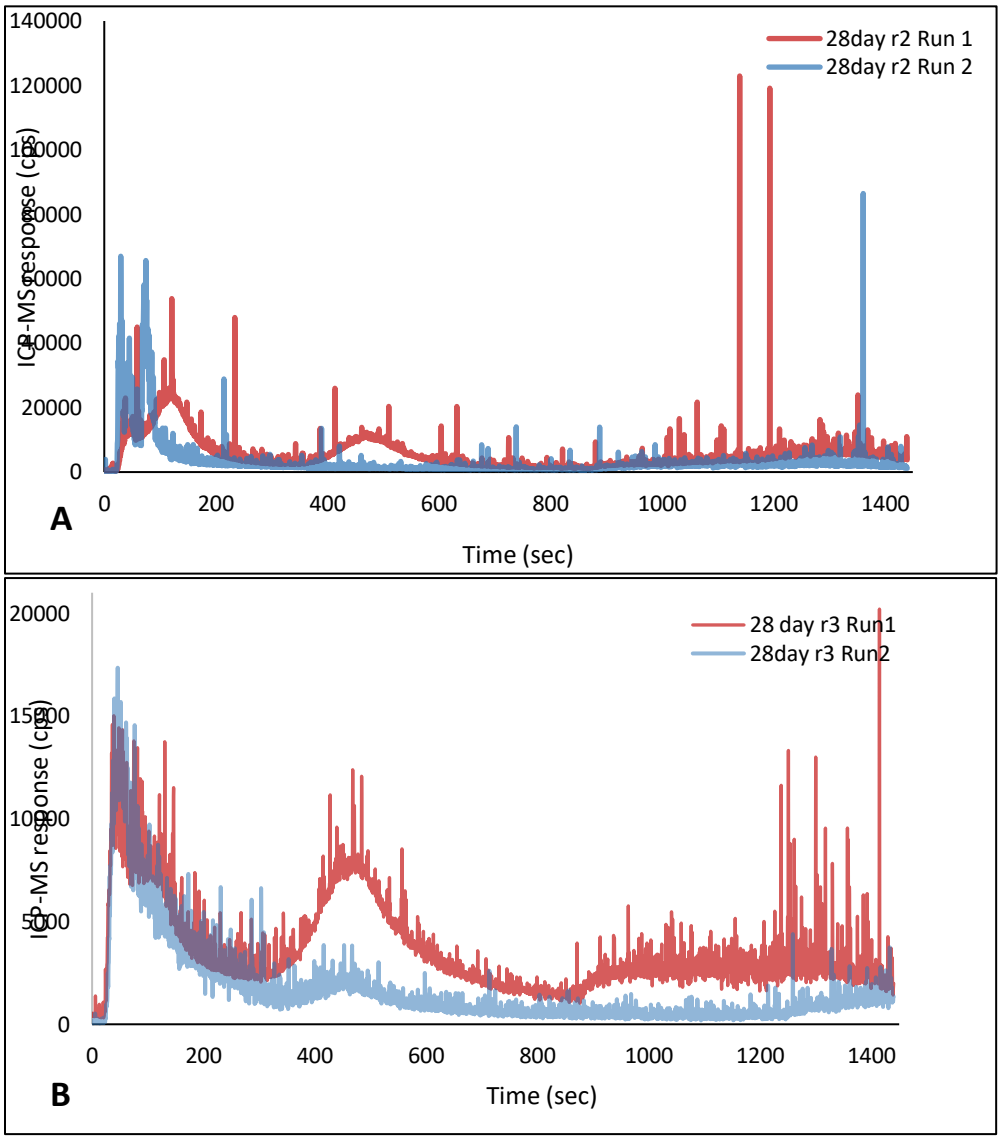

Figure 5.14. FFF-ICP-MS factograms of *L. variegatus* tissue exposed to 100 mg/kg d.w. sediment. Examples of a loss in sensitivity for 28 day samples – replicate 2 (A) and 3 (B).

In an attempt to improve ICP-MS sensitivity, carrier fluids listed in table 5.2 (NaN_3 and FL-70 surfactant) were diluted by a factor of 2.5 in MilliQ water in addition to a flushing of the ICP-MS instrument. A gold NP (12 nm, NanoComposix) was also introduced to *L. variegatus* samples in order to monitor ICP-MS sensitivity. MilliQ water samples including a mixture of AuNP (0.05 mg/L) and NM-300K (0.1 mg/L) demonstrated the different elution times of each NP; approximately 690 seconds and 960 seconds, respectively (figure 5.15). AuNP recorded a peak of approximately 40000 cps while NM-300K recorded a peak of approximately 36000 cps (figure 5.15). A longer elution time

was recorded for NM-300K than in previous factograms due to the dilution of carrier fluids. *L. variegatus* samples (28 day replicates 2 and 3) were then reanalysed with the inclusion of an AuNP standard (0.05 mg/L) and diluted carrier fluids, however, no AgNP peak was detected (as displayed for 28 day replicate 2, figure 5.16) Despite the absence of AgNP peaks in the *L. variegatus* sample, a clear AuNP peak was recorded (figure 5.16), with an intensity comparable to the peak recorded for AuNP in MilliQ water (40000 cps, figure 5.15), suggesting that no loss in sensitivity had been encountered.

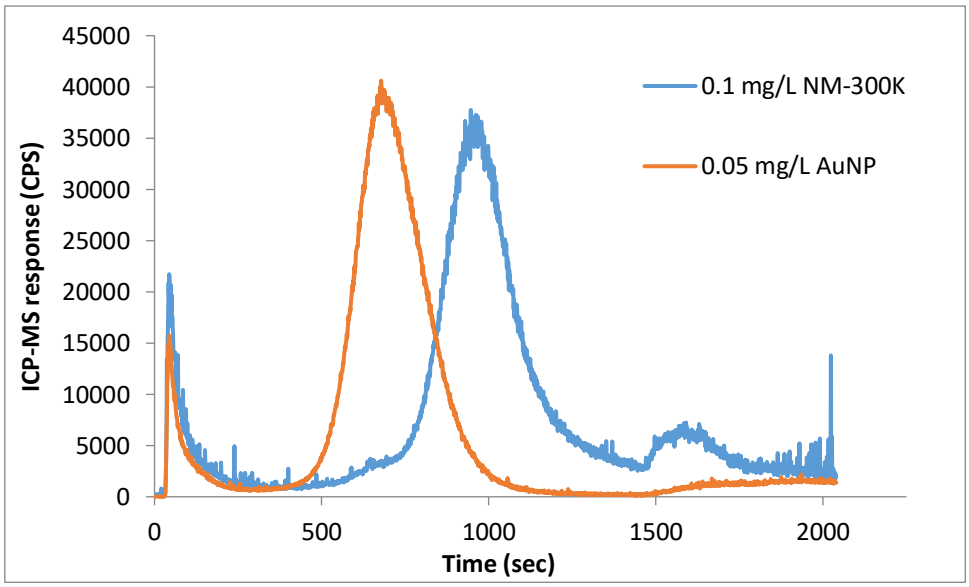

Figure 5.15. FFF-ICP-MS factograms of an AuNP (0.05 mg/L) and NM-300K (0.1mg/L) suspension in MilliQ water ran using diluted carrier fluids, n=1.

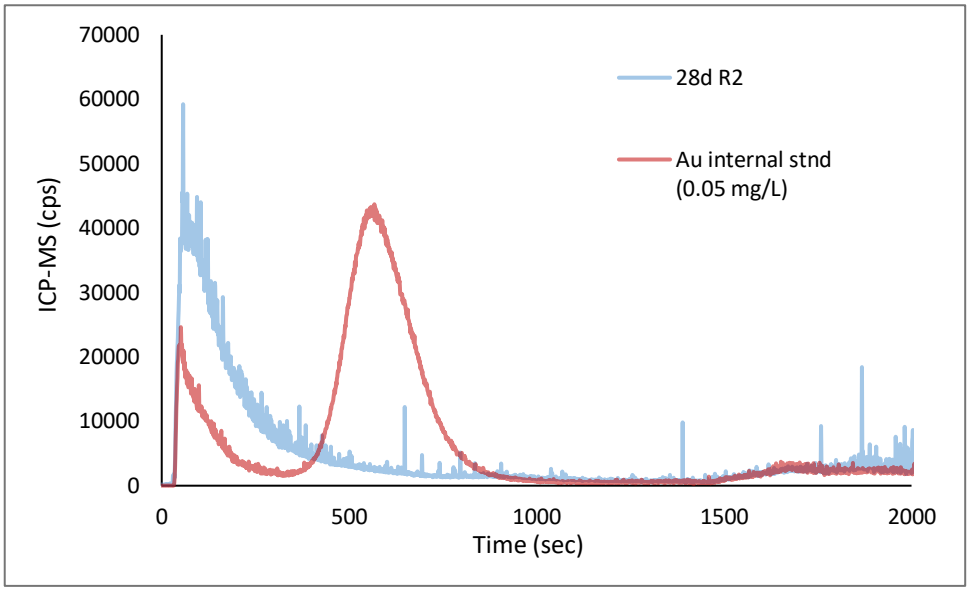

Figure 5.16. FFF-ICP-MS factogram of *L. variegatus* sample (day 28, r2) including an AuNP internal standard (0.05 mg/L) and ran with diluted carrier fluids. n=1

5.4 Discussion

5.4.1 NM-300K and NM-104 toxicity in relation to predicted environmental concentrations

Based on results obtained within this study, the hazard posed by NM-300K and NM-104 spiked sediments towards *L. variegatus* appears to be low. Whilst current environmental models forecast a seemingly sharp and constant increase in the predicted concentrations of NPs within sediments, these are anticipated to be within the low mg/kg or, in most instances within the µg/ kg or ng/kg sediment range.

Current models by Gottschalk *et al* (2009) based upon: worldwide annual production, potential release pathways, dissolution data, deposition and sedimentation behaviour, and ecotoxicological data, predict AgNP concentrations to be 2.2 µg/kg and 952 ng/kg sediment for the United States and Europe, respectively. Whilst toxic effects were exhibited within *L. variegatus* exposed to NM-300K spiked sediments at concentrations of 666.66 and 1333.33 mg/kg dry sediment as part of this investigation, these concentrations represent an apparent overestimation (in excess of 700,000 times the predicted value) of the above-mentioned European sediment concentrations.

Although the predicted sediment concentrations for TiO_2NP; 0.6 mg/kg (U.S) and 358 µg/kg (Europe) sediment are higher, owing to their anticipated greater worldwide production (Gottschalk *et al,* 2009); they still only represent a small fraction of the concentrations used within this investigation. A concentration of 1333.33 mg/kg dry sediment NM-104 was unable to evoke 100% *L. variegatus* mortality or any significant changes in biomass despite being over 3,500 times the predicted European sediment concentration according to Gootschalk *et al* (2009).

Currently, carbon nanotubes are the only NP for which a comparative evaluation of predicted sediment concentrations can be performed. Although the Gottschalk *et al* (2009) model is useful, given the lack of substantial data currently available, it is the only existing environmental model for Ag and TiO_2NPs in sediments and thus no comparisons can be made for the resultant predicted environmental concentrations (PEC). Whilst such PEC values should therefore be treated with a degree of caution, it is still evident that the sediment concentrations of NPs used within this investigation may be considered unrealistic in an environmental context. Nonetheless, the collection of such

data still provides a useful insight towards an otherwise overlooked area of NP ecotoxicology and may assist with the development of future environmental models.

5.4.2 Losses in L. variegatus numbers

As worm survival was only monitored following the full 28 days of exposure, identifying a specific time frame in which *L. variegatus* mortality occurred (i.e. in the case of 1333.33 mg/kg NM-300K where 100% mortality was recorded) was not possible. Although not required by OECD 225, sediment tests may be improved by intermittently assessing worm numbers (e.g. every 3-4 days) to approximately quantify if and when mortality has occurred. However a non-destructive approach would be advised (i.e. the application of heat to test vessels rather than sediment sieving which is more commonly used for sediment toxicity tests within the literature (Landrum *et al,* 2002; Higgins *et al,* 2007; Leppänen and Kukkonen, 1998).

Worms recovered from 666.66 mg/kg NM-300K and 1333.33 mg/kg NM-104 sediments were, predominantly, fragmented or whole worms with signs of regeneration and on average, only 12.3 and 11 worms were recovered, respectively. Damage incurred via threatening stimuli is usually thought to evoke autotomy responses from *L. variegatus,* whereby rapid fragmentation is followed by the regeneration of missing head or tail segments (Lesiuk and Drewes, 1999). It is possible that in 666.66 mg/kg and 1333.33 mg/kg exposures, *L. variegatus* accumulated higher levels of AgNPs, leading to the adaptive response of fragmentation and regeneration (potentially discarding tissue with high Ag burdens).

Lead-contaminated sediments have previously been shown to inhibit the regenerative capabilities of fragmented *L. variegatus* segments (Sardo *et al*, 2011). With this in consideration, it is possible that worm fragmentation may have occurred during exposure (either naturally or as a consequence of NP exposure), following which some fragments experienced regeneration whilst others either died or had their regeneration inhibited as a consequence of NP-contaminated sediments. It is possible to investigate *L. variegatus* regeneration according to Sardo *et al* (2011), whereby the growth of worms, cut into equal sections were measured following exposure to lead contaminated sediment and water. The effect of contaminants on *L. variegatus* regeneration capacity

is seen as a more sensitive endpoint than mortality and thus may be a useful test parameter within nano-ecotoxicology.

5.4.3 Changes in L. variegatus biomass

L. variegatus regenerate via morphallaxis, whereby existing body parts or tissue are transformed to newly organised structures with very little new cell growth Martinez *et al,* (2004). As regeneration is not inextricably linked with an increase in mass, an observed loss in dry biomass for worms recovered from 666.66 mg/kg NM-300K sediments (in comparison to control sediments) is likely to be a result of worm mortality. A potential inhibition of feeding behaviour may also account for losses in size and mass and has been previously demonstrated in phenanthrene-contaminated sediments (Lotufo *et al,* 1997). Leppänen and Kukkoen (1998) suggest that larger worms reproduce more frequently, thus a potential inhibition of feeding may be linked to a loss in reproduction and lower worm numbers. Despite an apparent decline in biomass following exposure to NM-104 contaminated sediments, none were found to be significant (although an unexpected increase in biomass was recorded for worms exposed to 83.33 mg/kg NM-104) suggesting no inhibition of feeding behaviour occurred.

Quantification of feeding behaviours were not implemented as part of this investigation, as OECD formulated sediments do not permit an adequate recovery of faecal pellets. However, introducing an additional sand layer on top of sediments as described by Leppänen and Kukkoen (1998), from which faecal pellets can be collected, could be considered as a useful additional endpoint in future NP-spiked sediment tests.

5.4.4 Effects of sub-lethal exposure on L. variegatus behaviour

Within the first 48 hours of exposure, standard burrowing behaviour was observed for *L. variegatus* in all test and control vessels (i.e. head segment within sediment, tail occupying the water column for gas exchange). Therefore, the low levels of toxicity observed cannot be attributed to sediment avoidance and a consequential lack of association with sediment-bound NM-300K and NM-104. Although little toxic effect was

witnessed for *L. variegatus* in NP-contaminated sediments as part of this study, their ability to exhibit standard burrowing behaviour and thus still initiate sediment bioturbation could have wider implications in an environmental context. Bioturbation of sediments re-suspends particulate matter in the water column and thus increases the availability of sediment-associated contaminants, (such as NPs) to alternate freshwater species.

Although only *L. variegatus* were used within this research, it is important to consider that the toxicity of metallic NPs can vary according to freshwater species (Griffit *et al,* 2008), whilst the actions of *L. variegatus* could potentially offer an alternate route of NP exposure to said species. Filter feeders such as daphnid species are particularly susceptible to NP exposure, e.g. *D. pulex* are thought to filter approximately 0.4 ml of water per hour (depending on environmental conditions) (Buikema, 1973). Furthermore, toxicity thresholds tend to be lower in comparison to *L. variegatus* (i.e. concentrations as low as 2 µg/L AgNP have been found to initiate 100% *D. magna* mortality in 24 hour exposures – Park and Choi, 2010).

A sub-lethal concentration of 166.66 mg/kg d.w. sediment NM-300K and NM-104 used within behavioural tests still represented a sizeable overestimate of potential environmental concentrations (section 5.4.1), however no significantly detrimental effects were recorded for either body reversal or helical swimming behaviours. The ability of *L. variegatus* to proficiently exhibit behavioural responses, vital for the avoidance of predation, despite the presence of a high sediment concentration of NPs further underlines the low toxicity of NM-300K and NM-104.

5.4.5 Comparison of sediment toxicity data

Due to the novelty of this research, no data are currently available for the comparison of Ag and TiO_2NP sediment toxicity towards *L. variegatus.* However, in relation to bulk-sized silver, Hirsch (1998) observed silver contaminated sediments (silver sulphide, 444 mg/kg) to pose very little toxic threat towards *L. variegatus*, despite evidence of accumulation within worms. Additionally, using an alternate, marine benthic worm species (*Arenicola marina),* Galloway *et al* (2010) measured both cellular and DNA damage following 10 days of TiO_2NP-spiked sediment exposure, recording a lowest

observed effect concentration (LOEC) of 1g/kg, a similar figure to the LOEC measured for *L. variegatus* within this study (1.33 g/kg NM-104).

Carbon nanoparticles, such as fullerenes (C_{60}) and carbon nanotubes (CNT) represent the most studied NPs within *L. variegatus* sediment toxicity testing (Pakarinen *et al,* 2011; Petersen *et al,* 2008). Similarly to this investigation, Pakarinen *et al* (2011) witnessed no changes in the burrowing behaviour, survival or reproduction of *L. variegatus* despite exposure to sediments contaminated with concentrations of fullerenes (10 and 50 mg/kg sediments) far exceeding PEC values estimated by Gottschalk *et al* (2009). Additionally, no mortality was observed in *L. variegatus* exposed to sediments containing 0.3 and 0.03 mg/kg multi-walled and single-walled carbon nanotubes, respectively by Petersen *et al* (2008).

5.4.6 Applicability of OECD 225 protocol for NP testing

The sediment-water *Lumbriculus* toxicity test using spiked sediment as outlined in OECD test guideline 225 was found to be applicable for the testing of NPs within this investigation. Deviations from the guidelines were not specifically designed to accommodate NPs but taken from a practical perspective. Both OECD formulated sediment and medium supported *L. variegatus* sufficiently, while testing met the requirements of validity stipulated within the protocol (all control sediment exposures recorded a >1.8 factor increase in worm numbers while water quality parameters were met).

The spiking of NPs represented the largest deviation from the OECD 225 guidelines. As outlined earlier, OECD 225 advises a bulk method of NP application whereby a dry sand portion is spiked, dried and added to the remaining sediment components. Alternatively within this study, NPs were added directly to wet sediment of each test vessel. Although this approach was adopted to maximise both time and equipment available, environmental relevance was also taken into consideration. A homogenous mixture of NPs within sediments provides a consistent exposure scenario for *L. variegatus*, whereby each worm is, in theory, likely to come into contact with and ingest approximately the same amount of sediment bound NPs. Whilst such a scenario might be useful for the purposes of data collection, it is unlikely to occur naturally within the

environment. NPs are most likely to be introduced to freshwater systems via the overlying water column (from both point and diffuse sources), following which sedimentation and deposition to the sediment surface is anticipated. Under such a scenario, *L. variegatus* are more likely to encounter NPs within the top layer of sediments (their natural habitat) where; by the process of bioturbation they will inevitably mix sediment associated NPs from the surface to deeper sediment layers. The creation of a well homogenised sediment potentially by-passes the above described *L. variegatus* - contaminated sediment interaction and thus would limit NP exposure. Direct 'wet' spiking has proved to produce more reproducible results, however Kühnel and Nickel (2014) also state that this method may not be suitable for all NPs (as the energy input required for dispersion prior to spiking may alter the surface of NPs).

5.4.7 Alternate NP exposure route

NPs and other potential contaminants are typically spiked directly to sediments within ecotoxicological research (Pakarinen *et al,* 2011, Galloway *et al,* 2010, Wang *et al,* 2014) and while this approach generally provides a reliable and reproducible means of generating data, it does not necessarily represent the most realistic NP exposure scenario for benthic organisms. By introducing NM-300K via the water column within sediment exposures, this study attempted to increase environmental relevance and in doing so, represented the largest deviation from the OECD 225 protocol. However, a >1.8 factor increase in worm numbers was not recorded for control groups, indicating poor worm health and a failure of OECD 225 test requirements. Accordingly, poor survival in NM-300K treated groups (observed especially at 5 mg/L and above) cannot be attributed to AgNP alone (despite results indicating a concentration dependent increase in *L. variegatus* mortality). The introduction of contaminants to overlying water have been implemented by Rosa *et al* (2015), however still represent a novel route of exposure, while no studies to date have implemented this approach in relation to NPs. Given the various routes through which NPs are likely to be introduced to the environment, the testing of NPs via the water column remain a viable option for ecotoxicological research.

5.4.8 Influence of NOM on NM-300K toxicity

As outlined in section 5.3.7, worms in 2% NOM content sediments (no NPs) were expected to more than double in number following 28 days of exposure, as witnessed in sections; 5.3.3, 5.3.4 and 5.3.5. Despite identical exposure conditions, i.e. sediment composition and production, overlying water composition and quantity, and storage worm numbers remained similar to the original number of worms added. Although the worms used in this investigation were stored and prepared in an identical manner (i.e. synchronised two weeks prior to exposure, ensuring a similar size and life stage), they were from a new culture – which represented the only deviation from previous exposures. An apparent poor state of *L. variegatus* health was therefore the most likely cause of the unexpected mortality incurred.

Despite unexpected mortalities, studies investigating the influence of the NOM content of sediment upon NM-300K toxicity (section 5.3.7) demonstrated that an increase in NOM content reduced NM-300K toxicity to *L. variegatus* when organism numbers and biomass were used to measure toxicity. As NOM concentrations in the environment are typically present in orders of magnitude far greater than NPs, it is likely that they will modify their properties and behaviour (Lowry *et al,* 2012). These authors hypothesise that the interaction of NOM with NPs may lead to the "masking" of NP effects, either directly via surface coating or through the minimisation of dissolution, however, quantification of this is difficult given the complex nature of sediments.

Studies investigating sediment NOM content upon AgNP behaviour (and toxicity) are scarce, however, Coutris *et al* (2012) revealed AgNPs to be less mobile in organic matter rich soils in comparison to soils with low organic matter. Furthermore, Shoults-Wilson *et al* (2011) witnessed significantly greater accumulation of AgNPs in the earthworm, *Eisenia fetida* when exposed in a sandy loam soil (OM content 1.77%) as opposed to an artificial soil (OM content of 7.65%). While Waalewjin-Kool *et al* (2014) anticipated a correlation between increasing soil OM content (2.37% - 14.7%) and toxicity of ZnO NP towards the arthropod *Folsomia candida* (hypothesising reduced dissolution and bioavailability in the presence of greater OM content), no such relationship was established (despite being established for $ZnCl_2$). Increased soil OM was in fact found to increase ZnO NP toxicity in their study and was found to be linked to greater rates of Zn dissolution. Waalewjin-Kool *et al* (2014) emphasise that increased dissolution may have

been linked to alternate soil properties (i.e. lower pH of high OM content soils), however, they also hypothesise that increased OM may have increased dissolution via de-agglomeration. Although these findings contradict the general consensus within the literature, they highlight the complex relationship between NPs and OM within soils/sediments and the need for further investigation.

While increased NOM content within sediments may have led to limited NM-300K dissolution within this study, the dissolution of NM-300K has previously been found to be relatively low (3-4% chapter 2) and found to not to be fully accountable for the toxicity exhibited by NM-300K towards *L. variegatus* (chapter 3). Using increasing peat contents in OECD 225 formulated sediments, Groh *et al,* (2010) observed decreasing accumulation of PCBs in *L. variegatus*¸ while *L. variegatus* in un-spiked sediments experienced greater growth and reproduction in higher OM sediments. Within this study, it is more likely that NOM sorption to NM-300K limited its bioaccessibility, and hence accumulation in and toxicity towards *L. variegatus.*

Studies using alternate species such as algae, Van Hoecke *et al* (2010) and *D. magna* Gao *et al,* (2012) indicate that increased organic matter content can reduce toxicity in water (described in greater detail in section 3.4.2), however, understanding the influence of NOM in sediment is less understood. Further studies measuring the uptake of NM-300K by *L. variegatus* (via ICP-MS) in sediments with varying NOM content could be conducted to explore this hypothesis. This investigation is the first to study the effect of sediment NOM content on AgNP toxicity. As sediments are potential sinks for NPs, understanding how their characteristics may influence NP behaviour and toxicity in the environment is of upmost importance.

5.4.9 FFF-ICP-MS

FFF-ICP-MS analysis was selected within this research due to its ability to confer information related to both NP size and composition. Although this technique has been successfully employed to investigate NPs within *L. variegatus* tissue following sediment exposures (Poda *et al,* 2011), data collected within this investigation proved inconclusive.

FFF-ICP-MS data confirmed the accumulation of NM-300K in sediment exposed *L. variegatus* (100 mg/kg d.w. sediment in samples after 3, 10, 18 and 28 days), limitations in FFF-ICP-MS methodologies meant no discernible patterns could be clarified between exposure periods, and a complete quantification of NM-300K within *L. variegatus* tissue could not be achieved.

Using FFF-ICP-MS, Poda *et al* (2011) found PVP coated AgNPs (31 nm) to have a retention time of approximately 500 seconds in water. However, in *L. variegatus* tissue (exposed to AgNP-spiked sediments for 28 days) a retention time of approximately 600 seconds was recorded, representing a particle size increase to 46nm. This increase in size was speculated to be the result of biological molecules/proteins coating AgNP surfaces, or the removal of PVP coating (due to abiotic reactions in sediment) leading to AgNP aggregation/agglomeration. While the data set is incomplete within this investigation, successful FFF-ICP-MS measurements for NM-300K in *L. variegatus* tissue were made and NM-300K NPs had approximately the same retention time as observed in MilliQ water (470 seconds). These results suggest that NM-300K (which were found to have an average size of 15.16 nm in MilliQ water via TEM– section 2.3.3) did not experience a dramatic change in size (as observed by Poda *et al,* 2011) following accumulation by *L. variegatus.*

Some changes in NM-300K size within *L. variegatus* tissue was anticipated over the course of sediment exposure and in comparison to NM-300K suspensions in MilliQ water (i.e. due to potential coating by sediment, organic matter or biological material). However, it is important to consider fundamental differences between experimental factors employed within this investigation and that of Poda *et al* (2011) – namely the AgNPs and sediment used within exposures. It is possible that NM-300K - which were not coated, but dispersed within Tween 20, remained more stable than the AgNPs used by Poda *et al* (2011), which potentially had their PVP coating removed during sediment exposures (effecting their interaction with sediments and biological material). Meanwhile, Poda *et al* (2011) employed a natural sediment, which due to likely differences in pH, organic matter content and composition to that of the formulated sediment used within this investigation, may have influenced AgNP fate, behaviour and ultimately size within *L. variegatus.*

Data obtained demonstrate that many *L. variegatus* samples were devoid of NM-300K peaks during FFF-ICP-MS analysis. FFF-ICP-MS has a detection limit of approximately 5 µg/L for Ag (Mitrano *et al,* 2012). While it is conceivable that the concentration of NM-300K within the aforementioned *L. variegatus* samples may have been below this limit, the detection of large NM-300K peaks within a number of samples and the inability to reproduce such peaks make it impossible to confirm this hypothesis. Furthermore, despite the absence of NM-300K peaks, the presence of void peaks still suggests the presence of NPs within samples. Dissolved Ag is removed by the FFF-ICP-MS cross-flow and exits the channel before reaching the ICP-MS. Therefore, void peaks are likely to consist of small NPs or Ag associated with organic moieties (e.g. proteins) (Coleman *et al,* 2013). Coleman *et al* (2013) also detected very few AgNPs in *L. variegatus* tissue (also in the presence of void peaks), however, using the highly sensitive single particle ICP-MS (which can detect parts per trillion) it was possible to detect larger AgNP peaks.

Method development for FFF-ICP-MS can be challenging given the multitude of variants to consider (e.g. flow rates, membrane type and carrier fluid composition). Within the limited literature concerning FFF-ICP-MS of *L. variegatus* tissue, regenerated 10kDa cellulose membranes are the only used (Poda *et al,* 2011, Coleman *et al,* 2013). The same membrane type was used within this investigation and replaced approximately every 25 runs as recommended by Mitrano *et al* (2012). Given that the membrane conditions used within this investigation have been successfully implemented for FFF-ICP-MS analysis of *L. variegatus* tissue previously, carrier fluids were instead investigated in an attempt to improve sensitivity.

Again, the carrier fluids used within this investigation (NaN_3 and 0FL-70 surfactant) have previously been used for *L. variegatus* analysis – however their concentration was altered in an attempt to improve sensitivity (carrier fluids are known to cause salt formation on ICP-MS cones, leading to a loss in sensitivity over time) (Williams *et al,* 2011). Despite diluting the carrier fluids (NaN_3 and 0FL-70 surfactant) by a factor of 2.5 and conducting a full ICP-MS rinse between runs, no improvement in sensitivity was recorded. This suggests that a loss in sensitivity was related to the size separation stage (i.e. FFF) rather than the detection (ICP-MS) stage of the procedure. It must also be noted that increases in run times (as experienced with diluted solvents) can increase the

likelihood of NP/membrane interactions, resulting in a decline in recovery (Mitrano, 2012)

The ability of FFF-ICP to separate NPs according to size and to perform multi-element analysis was manipulated in order to monitor sensitivity with the inclusion of an AuNP to *L. variegatus* samples. These results provide further evidence to suggest that a loss in sensitivity was the result of the NP separation rather than their detection, as Au peaks remained unaltered in *L. variegatus* tissue samples (as compared to in MilliQ water), while no peaks were detected for Ag. AuNPs were introduced immediately prior to analysis and thus were unlikely to interact with (and be altered) by biological components within samples. It is more likely that NM-300K, following sediment exposure and accumulation in *L. variegatus* may have become altered or associated with sediment or biological material, which ultimately affected the efficiency of FFF separation over time. Although the inclusion of AuNPs was redundant in this scenario, this investigation is the first (in the context of *L. variegatus)* to explore the use of an internal standard to monitor FFF-ICP-MS sensitivity – which given the number of variables to consider, may be important in obtaining accurate and reproducible results.

The method employed for the digestion of *L. variegatus* within this investigation was based on that of Gray *et al* (2013), who found TMAH to successfully extract AgNP and AuNPs from *L. variegatus* tissue for use in SP-ICP-MS analysis. Based on the results of this investigation, TMAH was able to successfully extract NM-300K from *L. variegatus* tissue for FFF-ICP-MS analysis. However, alternate *L. variegatus* digestion methods used by Poda *et al* (2011) and Coleman *et al* (2013) include a centrifugation (and in the case of Coleman *et al,* 2013) a subsequent filtration (to 0.45 µm) stage to remove biological debris. Without centrifugation and/or filtration of digested samples, it is possible that biological debris adhered to membranes, impeding the diffusion of NPs back into the FFF channel and ultimately limiting their recovery. While TMAH digestion of *L. variegatus* has been successfully implemented by Gray *et al* (2013) for use with SP-ICP-MS analysis – this does not involve a membrane and thus avoids the potentially problematic tissue/NP/membrane interactions encountered with FFF-ICP-MS.

Based on the findings of this research, future recommendations for the analysis of NPs within *L. variegatus* tissue via FFF-ICP-MS include:

- The centrifugation and/or filtration of samples following digestion (with TMAH) in an attempt to remove biological debris and limit its adverse effect upon NP recovery within the FFF channel.
- Changing of FFF membranes on a regular basis. Although a membrane change is recommended for every ~25 runs (Mitrano *et al,* 2012), this could be altered to 15-20 in an attempt to limit the effects of biological debris and improve sensitivity (cost dependent, however).
- Avoiding the dilution of carrier fluids – the longer run times associated may give rise to increased membrane adhesion and poorer sensitivity.
- The inclusion of an internal standard of a known concentration to monitor ICP-MS sensitivity. Given the low concentration of NPs often used within FFF-ICP-MS analysis, small drifts in sensitivity could have great repercussions.
- Complementing FFF-ICP-MS analysis with the highly sensitive SP-ICP-MS technique – especially for instances where no peaks are detected using FFF-ICP-MS. Coleman *et al,* (2013) have previously demonstrated the ability of SP-ICP-MS to detect AgNPs in *L. variegatus* samples despite observing no peaks using FFF-ICP-MS. SP-ICP-MS was not used within this investigation as NM-300K was within the size detection limit for Ag - 11-20 nm (Lee *et al,* 2014).

5.5 Conclusion

NP-spiked sediment exposures within this investigation revealed both NM-300K and NM-104 to display relatively low toxicity towards *L. variegatus* in relation to reproduction and biomass, despite the high concentrations used (in relation to predicted concentrations). Deviations in sediment spiking techniques and mixing were made from OECD guideline 225 in an attempt to improve experimental efficiency and environmental relevance and to limit the adhesion of NPs to mixing equipment. Furthermore, a lower NOM content (2% rather than 5%) was also used to increase environmental relevancy and provide a more conservative estimate of NP toxicity within sediments. OECD guidelines were otherwise implemented as described and were found to successfully support *L. variegatus* (within controls) and be applicable for the testing of NPs. Despite poor worm health compromising results, the spiking of sediments via the water column represent a novel exposure route for testing with *L. variegatus* and are potentially a more environmentally relevant option in gauging sediment toxicity.

L. variegatus inhabit a wide range of environments and will thus experience sediments of differing composition. While NOM is widely acknowledged to influence NP fate and behaviour in the environment – a range of different NOM content sediments or soils are sparingly used within ecotoxicological testing of NPs. By altering the NOM content of sediments within this investigation, it was possible to exhibit its influence on toxicity, with increasing NOM content resulting in reduced toxicity. Analysis via FFF-ICP-MS confirmed the uptake of NM-300K during 28 day, sub-lethal exposures. After experiencing a loss in the sensitivity of readings, method analysis confirmed this to be a result of the separating process of NM-300K (FFF), rather than the detection (ICP). It is advised that a centrifugation and/or filtration of samples is performed prior to digestion in order to limit the adhesion of biological material to the FFF membrane.

Chapter 6: General discussion and conclusions

6.1 Overview of findings

This thesis has demonstrated the effect of two JRC representative-manufactured NPs, NM-300K (AgNP) and NM-104 (TiO_2NP) towards *L. variegatus* in (acute) water and (chronic) sediment exposures. While NM-300K displayed toxicity in relation to *L. variegatus* mortality, loss of biomass, inhibition of behavioural responses and the induction of antioxidant enzymes SOD and CAT – no such findings were evident for NM-104.

6.1.1 Correlating NP properties to toxicity

The toxicity of NPs are commonly associated with their physicochemical properties, which can undergo transformations within the environment and environmentally relevant media. From the data provided by various, complimentary sizing techniques (table 6.1), it is clear that NM-300K are considerably smaller and more stable than NM-104 in Milli-Q water and OECD medium. Although some aggregation/agglomeration was evident for NM-300K in OECD medium over time, NM-104 experienced far greater and more rapid aggregation/agglomeration in both Milli-Q water and OECD medium. These findings are in keeping with the available literature where NM-104 is commonly acknowledged to form large aggregates/agglomerates in Milli-Q water (Rasmussen *et al,* 2014) and ecotoxicological testing media (Faria *et al,* 2014, Nicolas *et al,* 2015 and Campos *et al,* 2013).

Table 6.1 Summary of mean characterisation data for NM-104 and NM-300K in Milli-Q water and OECD medium for 0, 24 and 96 hours. Grey boxes = no measurement made.

		NM-104				NM-300K			
Media	Time (hr)	HD (nm)	ZP (mV)	TEM (nm)	DCS (nm)	HD (nm)	ZP (mV)	TEM (nm)	DCS (nm)
Deionised H20	0	95.8	-16.6	159.06	221.4	57.2	-24.5	15.16	15.2
	24	98.7	22.2			58.2	-17.5		
	96	270.6	8.7			48.4	-29.4		
OECD	0	278.6	-0.75			66.7	-10.8	15	
	24	907.7	0.56			66.7	-16.3	16.44	
	96	3018.6	-11.87			78	-15.6		

Size is regarded to be a prominent factor in determining the toxicity of TiO_2NPs in the aquatic environment (Faria *et al,* 2014). Increases in size (due to aggregation/agglomeration) are likely to limit the total NP mass in the appropriate size-range for direct uptake in the water phase (Baun *et al,* 2008) (i.e. limiting bio-availability), while NPs are more likely to penetrate cells and evoke toxic responses. Furthermore, aggregation/agglomeration also limits toxicity by decreasing the reactive surface area of NPs. It is possible that despite high concentrations (up to 1.5 g/L), rapid aggregation of NM-104 in OECD medium led to limited uptake by *L. variegatus*, hence limiting toxicity. Faria *et al* (2014) reported NM-104 aggregates (hydrodynamic size of 1361.33nm – similar to those recorded in OECD medium, figure 6.1) at a concentration of 1 mg/ml to exhibit low bioavailability toward zebrafish embryos and only display marginal levels of toxicity in relation to growth.

While NM-104 may display limited toxicity directly in the water phase, it may have wider implications on energy transfer within the aquatic food web due to its strong tendency to aggregate/agglomerate. For example, Campos *et al* (2013) observed the aggregation of NM-104 (1mg/L and 10 mg/L) within *D. magna* culture medium (due to high ion content) and the interaction of NM-104 aggregates with algae to form large clusters. The subsequent sedimentation of NM-104-algae clusters dramatically limited the availability of algae to *D. magna* leading to adverse effects on reproduction and fitness. Aggregation/agglomeration of NM-104 and the formation of NM-104 clusters in the aquatic environment will lead to the settlement of edible particles from the water column. As non-selective feeders predominantly occupying sediments, *L. variegatus* are thus highly likely to encounter and ingest NM-104 aggregates and agglomerates. Within this investigation, exposure to NM-104-spiked sediments (where *L. variegatus* were

intimately associated with sediments for 28 days) still revealed low levels of toxicity (changes in *L. variegatus* numbers only recorded at 1333.33 mg/kg d.w. sediment NM-104).

Due to the high affinity for NM-104 to aggregate/agglomerate in environmental media, it is difficult to determine the toxicity of stable, well-dispersed NM-104 particles. Consequently, it is also difficult to decipher whether the low levels of toxicity observed for NM-104 towards *L. variegatus* were the result of aggregation/agglomeration (limiting NM-104 bioavailability, reactive surface area and potential for cell penetration) or whether the particles themselves exert little toxicity. Surface charge, as measured by zeta potential, is known to influence aggregation and thus, potentially toxicity. ZP data for NM-104 in OECD medium revealed relatively neutral values, particularly after 0 and 24 hours (table 6.1) – explaining the high levels of aggregation/agglomeration observed. As previously discussed, TiO_2NP ZP can differ significantly in accordance with pH. The JRC (Rasmussen *et al,* 2014) observed NM-104 to form unstable suspensions between pH 5-9 and possess an isoelectric point (pH at which surface charge is globally neutral) of 8.2, figure 6.1.

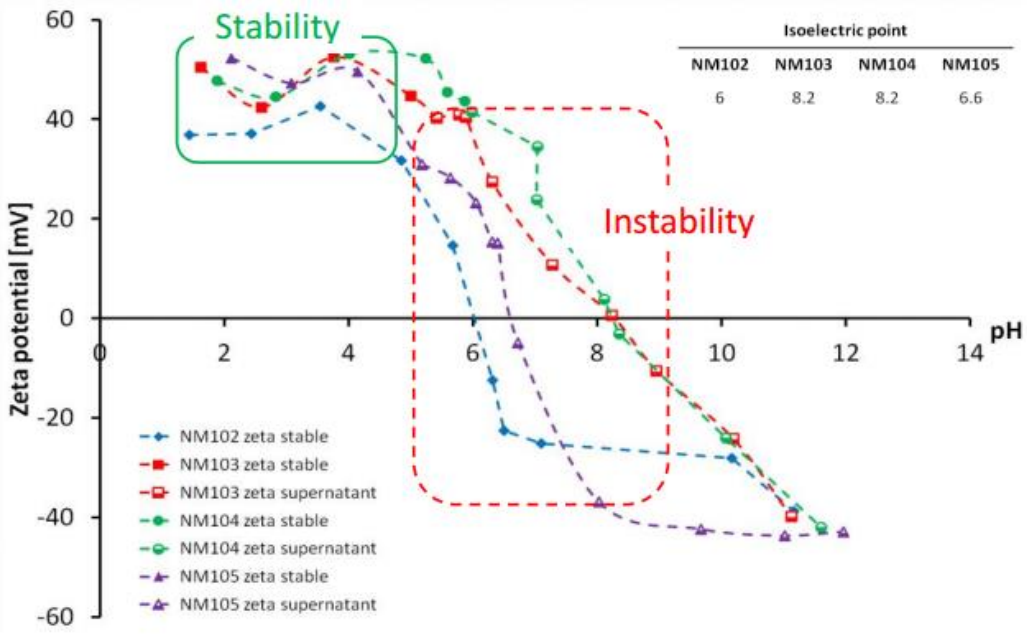

Figure 6.1 Zeta potential as a function of pH for NM-104 (green line) in constant ionic strength aqueous media. (Soure: Rasmussen *et al,* 2014).

With a pH of 7.7, OECD medium used within this investigation led to the formation of a neutral NM-104 surface change and facilitated their aggregation/agglomeration. By increasing the pH range (i.e. to values below pH 5 and above pH 9), NM-104 may become more stable, bioavailable and hence, more toxic towards *L. variegatus.* However, such extremes of pH are seldom experienced within freshwater ecosystems, with typical values ranging from 6.5-8 (Flanagan, 1986). As these figures lie within the pH range deemed to create unstable NM-104 suspensions (figure 6.1, Rasmussen *et al,* 2014), it could be argued that the ecotoxicological testing of NM-104 aggregates/agglomerates is more environmentally relevant for *L. variegatus* and other freshwater species, as opposed to well dispersed particles.

Comparatively, NM-300K particles retained a relatively negative surface charge in OECD medium and consequently experienced less aggregation/agglomeration as displayed in the hydrodynamic diameter and TEM data collected (table 6.1). Characterisation data obtained for NM-300K in OECD medium contribute toward the theory that increased stability and dispersion of NPs increase their bioavailability, reactive surface area, potential for cell penetration and ultimately, their toxic potential toward environmental species.

The fate and behaviour of NM-104 and NM-300K in OECD medium undoubtedly influenced toxicity toward *L. variegatus* within this investigation. However, the marked differences in toxicity observed between NM-104 and NM-300K are still likely to be attributed to elemental composition - as 'bulk-form' TiO_2 is widely regarded to be inert and Ag, highly toxic. The large surface area of metal-based NPs often facilitates the release of metal ions into solution, which in the case of Ag, are highly toxic due to their ability to inhibit enzymes and membrane transporters (Shipelin *et al,* 2015). Consequently, it is not always clear whether observed toxicity is the result of unique nano-related properties (e.g. size, shape, surface charge, ability to bind to biological sites) or the release of ions via dissolution. Low levels of silver dissolution within OECD medium (<4%) concurred with the available literate in regards to NM-300K in environmental media (Cupi *et al,* 2015; Mallevre *et al,* 2014). Using these data, it is possible to conclude that the release of Ag^+ was only partially responsible for the toxicity exhibited by NM-300K towards *L. variegatus* in OECD medium (in relation to mortality).

Similarly, Ribeiro *et al* (2015) concluded that the slow release of Ag^+ from NM-300K could not fully explain toxicity towards *Enchytraeus crypticus* (in soil), citing different mechanistic pathways between NM-300K and $AgNO_3$ (metallothione, total glutathione and CAT increases associated with AgNP exposure, lipid peroxidation, GST, and GPx associated with $AgNO_3$ exposure). Bertrand *et al* (2016) were also unable to attribute all observed toxicity toward *Scrobicularia plana* (in relation to oxidative stress, cell damage and energetic levels) to the released Ag^+ fraction of NM-300K in seawater. Conversely, Schlich *et al* (2012) concluded that Ag^+ was responsible for the inhibition of *Eisenia andrei* reproduction following NM-300K exposure (in soil), while van der Ploeg *et al* (2014) similarly suggested that the release of Ag^+ from NM-300K was responsible for growth and reproduction inhibition in *Lumbriculus rubellus* (in soil -although without measuring dissolution).

While conflicting assumptions exist within the literature as to the source of NM-300K toxicity, there is considerable evidence to suggest that released Ag^+ account for at least part of the toxicity. It is also important to consider the combined effects of AgNP and released Ag^+ in a mode of action referred to as the "Trojan horse effect", whereby AgNPs are able to penetrate biological barriers (due to their size), accumulate in cells and release Ag^+ ions in high, localised concentrations and hypothesised to cause toxicity (Shipelin *et al,* 2015). Furthermore, conflicting dissolution kinetics may arise in accordance with test systems (e.g. sediments, soil, freshwater, seawater) and abiotic factors (pH, ionic strength, NOM content and temperature) which may ultimately influence modes of toxicity.

The determination of NP properties within this research underline the importance of characterisation within testing medium, with both NM-300K and (to a greater extent) NM-104 undergoing transformations upon their introduction to OECD medium. Differences between NM-300K and NM-104 properties (and hence, toxicity towards *L. variegatus*) also emphasise the need for a case-by case approach for NP risk assessments. Additionally, while it may be appropriate to treat the risk associated with NM-104 as similar to that of its bulk counterpart (due to high levels of aggregation/agglomeration and associated low-levels of toxicity, the same cannot be applied to NM-300K). Through the investigation of dissolution properties of NM-300K,

it is clear a nano-specific toxicity exists towards *L. variegatus* in OECD medium, suggesting that its risk should be assessed differently to that of Ag^+ alone.

6.1.2 Aquatic toxicity

Within OECD medium, NM-300K caused mortality towards *L. variegatus* (LC_{50} value of 569.7 µg/L), while sub-lethal exposures (200 µg/L) inhibited behavioural responses (helical swimming and body reversal) and initiated time-dependent increases in SOD content and CAT activity. Conversely, NM-104 aquatic exposures caused no mortality (0-1.5 g/L), nor changes in behaviour or antioxidant enzyme levels.

Despite being one of the few oligochaete species capable of swimming, the distinctive behavioural traits of *L. variegatus* are scarcely used within the ecotoxicological testing of NPs. Both body reversal and helical swimming are essential in relation to *L. variegatus* rapid dispersal and avoidance of predation, while locomotive behaviours are also innately linked to successful sexual reproduction, foraging ability and general orientation to environmental cues (Drewes, 1997). *L. variegatus* rely on these behaviours to perform essential ecosystem services such as accelerating detrital decomposition (Wallace and Webster, 1996), releasing nutrients via foraging, feeding and excretion and facilitating nutrient transfer. Furthermore, the inhibition of *L. variegatus* behaviour would limit their abilities as predators and their ability to escape predation themselves, potentially leading to major shifts within the freshwater aquatic food chain.

Given the importance of *L. variegatus* behaviours, the inhibition of both helical swimming and body reversal within this investigation suggest that sub-lethal NM-300K exposure (0.05, 0.1 and 0.2 mg/L) would have severe implications upon ecosystem functioning. For example, the inhibition of escape behaviours could result in increased predation of *L. variegatus* – subsequently inhibiting their ecological role in sediment bioturbation and nutrient cycling in freshwater environments. Meanwhile, NM-104 exposures had no effect upon *L. variegatus* behaviour, either directly (due to uptake and assimilation) or indirectly, due to the adhesion of sedimented NM-104 aggregates to the exoskeleton.

Behavioural assays proved to be a valuable, non-destructive endpoint within this investigation and displayed similar patterns in comparison to antioxidant enzyme data, over time. As outlined in table 6.2, both behavioural responses deteriorated over time up to 24 hours, where extremely poor responses were recorded (i.e. almost no response to tactile stimulation). However, after 48 and 72 hours, behaviour generally improved (especially in the case of helical swimming, where responses were found to be significantly similar to those of controls). An apparent loss in behaviour after 24 hours coincided with an increase in both CAT activity and SOD content, which after 48 hours, and 72 hours, respectively returned to levels significantly similar to that of controls.

Based on the results of this investigation, *L. variegatus* experienced the greatest toxicity within 24 hours' exposure to NM-300K. Mechanisms that protect cells from oxidative stress, such as endogenous antioxidants (including SOD and CAT) consume a considerable amount of energy when activated for prolonged periods of time (Poljsak *et al,* 2013). The activity of SOD increased following only 2 hours (0.2 mg/L) NM-300K exposure, and while increased levels were not recorded after 6 hours, it is possible that increases were experienced between 6-24 hours. Similarly, CAT may have experienced increases between 6-24 hours, despite displaying no increase after 2 or 6 hours (table 6.2).

Table 6.2 Changes in SOD content, CAT activity, body reversal (BR) and helical swimming (HS) of *L. variegatus* exposed to 0.2 mg/L NM-300K for 2, 6, 24, 48 and 72 hours in OECD medium. ↑ = increase in SOD/CAT in relation to control, − = no increase in SOD/CAT in relation to control, BR and HS data expressed as % success rate, *= no significant difference from control ($p<0.05$).

Endpoint	Time (hours)				
	2	6	24	48	72
SOD content	↑	–	↑	↑	–
CAT activity	–	–	↑	–	–
BR (%)	57.7	44.44	6.66	48.8	33.33
HS (%)	42.22	28.88	4.44	62.22*	72.22*

While further SOD/CAT testing between 6-24 hours would be needed to investigate this hypothesis, it is possible that a considerable amount of *L. variegatus* energy was allocated to antioxidant production in the first 24 hours of NM-300K exposure. It could

be hypothesised that energy, which would otherwise be allocated to behavioural responses, was instead contributed towards the production of SOD and CAT.

The return of CAT and SOD to levels significantly similar to those recorded for control *L. variegatus* after 24 and 48 hours respectively, coincided with a general improvement in behavioural responses (body reversal proved to be more variable, while helical swimming greatly improved, compared to 2-24 hour responses – table 6.2). These findings demonstrate the apparent ability of *L. variegatus* to recover following initial NM-300K mediated toxicity and provide further evidence towards an energy-allocation theory (i.e. after increases in SOD and CAT were able to offset toxicity, their production was reduced and energy allocations returned to normal, leading to an improvement in behavioural responses). A lack of lipid peroxidation discovered within this investigation provides further evidence to suggest that *L. variegatus* are able to offset the toxic effects of NM-300K in short term, aquatic exposures (however, additional parameters of oxidative damage could be measured to confirm this). Although *L. variegatus* could recover behavioural responses, the initial inhibition witnessed within 24 hours could still lead to an increase in predation within the environment.

Similar hypotheses have been drawn in relation to glyphosate and Roundup Ultra exposure by Contardo-Jara *et al* (2009) who suggested that energy required for the synthesis of GST (an isoenzyme responsible for catalysing the conjugation of reduced glutathione (GSH) to electrophilic substrates producing subtances that are less reactive) may occur at the expense of *L. variegatus* growth or reproduction. Although not considered within the research, measurements of growth and reproduction could be made in future (chronic) aquatic exposures to further investigate this theory.

O'Rourke et al (2015), conducted similar studies investigating *L. variegatus* behaviour and oxidative stress parameters in relation to ZnO NP with somewhat conflicting results. While detrimental effects upon behaviour (body reversal) were recorded following ZnO NP exposure for 96 hours (1.25-10 mg/L), no oxidative response (as measured by significant changes in GSH levels) were recorded after 0, 4, 8, 24, 48, 72 and 96 hours. O'Rourke *et al* (2015) indicated that while the test concentrations used were able to inhibit body reversal behaviour, higher concentrations may be required to evoke changes to GSH. Furthermore, O'Rourke *et al* (2015) also discuss the theory that antioxidant mechanisms are transient and vary according to species and chemicals

tested acknowledging that a wider range of oxidative stress biomarkers could have been employed. It is plausible that *L. variegatus* are more sensitive to AgNP than ZnO (despite the considerably lower concentration of NM-300K used within this study), while the inclusion of SOD and CAT may have uncovered similar oxidative and behavioural responses following ZnO NP exposure within the study of O'Rourke *et al* (2015).

6.1.3 Influence of abiotic factors

Although the presence of 5 mg/L SRHA did not cause a significant shift in the LC_{50} value generated for NM-300K in OECD medium, it had a profound effect upon sub-lethal toxicity towards *L. variegatus*. As discussed in section 6.1.2, a concentration of 0.2 mg/L NM-300K inhibited *L. variegatus* behaviours and initiated changes in SOD and CAT – however, following the inclusion of 5 mg/L SRHA to OECD medium, no such observations were made.

Based on the results of NM-300K characterisation within 5 mg/L SRHA OECD medium, it was hypothesised that SRHA inhibited a nano-specific element of NM-300K toxicity (either as a physical barrier or by reacting with potential ROS) as no inhibition of dissolution was observed. It is possible that the concentration of SRHA used within this investigation was not high enough to coat NM-300K particles to the extent where the dissolution was inhibited. Quantifying concentrations of humic substances in freshwater environments is challenging given their transient nature, however, Ferguson (2011) estimate oligotrophic waters to contain HA concentrations within a range of 0.5 – 80 mg/L. Based on these estimates, and in comparison to similar studies where considerably higher concentrations are often employed e.g. 10 mg/L (Fabrega *et al,* 2009), 100 mg/L (Yang *et al,* 2013) and 600 mg/L (Gunsolus *et al,* 2015), a concentration of 5 mg/L represents a moderately conservative estimate. Future studies using higher SRHA concentrations could be employed to assess whether an effect on dissolution is observed, potentially negating the toxic effect of NM-300K further.

Concentrations of NM-300K found to cause toxicity towards *L. variegatus* (lethal and sub-lethal) were considerably higher than the predicted environmental concentrations (PEC) for AgNPs in surface waters. Models constructed by Gottschalk *et al* (2009) produced a PEC of 0.764 ng/L for AgNP in European surface waters. The LC_{50} value for

NM-300K in OECD medium (no SRHA) represents a figure 650,000 times greater than this PEC, while sub-lethal effects upon behaviour and antioxidant enzymes were observed at concentrations approximately 65,000 and 260,000 times greater than the PEC value, respectively. While the accuracy of models is limited and the release of AgNPs will inevitably rise as nanotechnologies continue to grow, the concentrations observed to induce toxicity within this research seem unlikely in an environmental context. Furthermore, the toxic effects observed were seemingly negated by a somewhat conservative estimate of NOM concentrations. Therefore, it could be hypothesised that the hazard posed by NM-300K at environmentally relevant concentrations and in environmentally relevant conditions is low in relation to *L. variegatus.*

6.1.4 Sediment toxicity and applicability of OECD test guidelines

The OECD are undertaking a large effort to ensure that test guidelines are available for the regularity ecotoxicity testing of NPs. As part of the MARINA project, this research focused on the adaptation (in relation to NM-300K and NM-104) of OECD test guideline 225: Sediment-water *Lumbriculus* toxicity test using spiked sediment. As only minor adaptations were made in relation to NM-300K and NM-104 sediment testing within this investigation, OECD test guideline 225 was adjudged appropriate for use with NPs. Amongst the proposed adaptations, the reduction of the NOM component (peat) of sediments from 5% to 2% was also applied for other OECD test guidelines tested within the MARINA project. The reduction of NOM content within this investigation was justified in order to better replicate the NOM content of natural sediments, while a reduction in soil NOM (OECD 222) was similarly applied to reflect a larger area of agricultural conditions (Hund-Rinke *et al,* unpublished). Changes to 2% NOM were also observed in OECD 220 in order to facilitate the comparison of results between species and functions (Hund-Rinke *et al,* unpublished). The individual NP-spiking and mixing of replicates proposed for sediments in OECD 225 (as opposed to bulk spiking and mixing of sediments) was also recommended for soils in OECD 220, namely to reduce variation between replicates and ensure homogenous mixtures.

Although OECD test guidelines have previously been reviewed for use with NPs (OECD, 2009) and undergone development in relation to sample preparation and dosimetry (OECD, 2012a), specific guidance on individual toxicity tests have not been provided. By evaluating test guidelines individually through MARINA and this investigation, a better understanding of the scientific and practical limitations has been realised, allowing an efficient and co-ordinated collection of ecotoxicological data for NP exposures.

Similar to the results of aquatic exposures, NM-300K and (to a greater extent) NM-104 displayed low toxicity towards *L. variegatus* within sediment exposures, particularly in light of their respective PECs. Models by Gottschalk *et al* (2009) produced European PECs within the ng/kg and µg/kg sediment range for AgNPs and TiO_2NPs, respectively. Toxicity was only observed following exposure to high mg/kg concentrations for AgNP, while no toxicity was observed for NM-104 up to a concentration of 1.3g/kg sediment. These results strongly indicate that PECs of both NPs in sediment would be unlikely to have a negative impact upon *L. variegatus* and comply with the consensus of ecotoxicity data generated within MARINA using OECD test guidelines for various freshwater species (*Pseudokirchneriella subcapitata, D. magna, Danio rerio, Enchytraeus crypticus* and *Eisenia fetida)* (Hund-Rinke *et al,* unpublished). FFF-ICP-MS data revealed the presence of NM-300K in *L. variegatus* tissue following exposure in 100 mg/kg d.w sediment – ruling out a lack of uptake as an explanation for the low toxicity observed. Although FFF-ICP-MS research within this investigation became focused on method optimisation, future work with sediment exposed *L. variegatus* could provide valuable information related to NM-300K characteristics once ingested and which could potentially be used to infer any toxic effect (or absence of).

Although low toxicity was observed in relation to mortality and dry biomass (endpoints of interest for OECD 225), *L. variegatus* may have undergone similar sub-lethal stresses as observed in aquatic toxicity testing over the 28 day sediment exposure period. However, given the transient nature of antioxidant enzymes (as witnessed in aquatic exposures over 72 hours), any changes in antioxidant enzymes may have already occurred after 28 days. Using multiple time points (3, 7, 14 and 28 days) Wang *et al* (2014) found that the CAT activity of *L. variegatus* only increased after 14 days exposure to fullerene (C_{60}) – spiked sediments, returning to values similar to controls after 28 days. By incorporating additional assays (i.e. SOD and CAT) and multiple sampling time-

points into chronic sediment exposures (such as OECD 225), a more holistic understanding of NP toxicity could be achieved and could be considered for future experiments.

6.2 Recommendations and future research

Testing the applicability of current test guidelines for NP hazard assessment is of high societal importance. Sediment exposures within this research represent the first to evaluate the applicability of OECD 225 test guidelines for use with NPs and while this work has provided valuable information in regards to NM-300K and NM-104 sediment toxicity, key questions remain. Although the primary aim of OECD test guidelines 225 is to assess toxicity (and in the case of this investigation, to assess its suitability for NP testing), no information related to fate and behaviour can be deduced. Understanding fate and behaviour is integral in understanding the risk posed by NPs and ultimately ensuring the sustainable development of nanotechnologies.

While no validated analytical techniques are currently available for the detection and characterisation of NPs within sediments and other complex environmental media (Kühnel and Nickel, 2014), the increasing availability and application of FFF and sp-ICP-MS potentially offer a greater insight. Through its continuous separation of complex samples, FFF provides a continuous set of monodisperse NPs that can be readily characterised using an array of additional techniques (Baalousha and Lead, 2011). However, as experienced with *L. variegatus* tissue within this investigation, FFF is not without its limitations – most notably sample-membrane interactions that are cited to induce up to 50% losses in NP mass of samples (Hassellöv et al, 2009). SP-ICP-MS is similarly capable of detecting the size and ultra-trace concentration of NPs, however, like FFF-ICP-MS, is yet to be implemented for NP detection within sediments. By optimising techniques such as FFF and SP-ICP-MS for use with NPs in sediments, it would be possible to link NP characteristics in sediments to the toxicity observed through the use of protocols such as OECD test guideline 225. Additionally, more precise PEC concentrations could be measured, possibly increasing the relevancy of ecotoxicological research, while the efficiency of spiking techniques could be assessed.

Based on current PECs for Ag and TiO_2 NP and the toxicity data collected within this investigation, it is highly unlikely that NM-300K or NM-104 will pose a significant risk to *L. variegatus* in the environment. Sub-lethal effects (I.e. increases in SOD and CAT) were only observed at relatively high (and environmentally unrealistic) NM-300K concentrations and in the absence of NOM. Furthermore, *L. variegatus* were seemingly able to recover from sub-lethal effects over time. This research is the first to investigate the effects of AgNP and TiO_2 NP on the antioxidant defence system of *L. variegatus* and provides evidence to suggest that NM-300K induces oxidative stress (with no such findings for NM-104). While these results provide valuable information in relation to NM-300K toxicity, the antioxidant system works in a cooperative way (Winston et al, 1998) and the testing of isolated assays may not provide a holistic understanding of *L. variegatus'* response to NM-300K exposure. Methodologies (based on the detection of ROS) exist for the assessment of total antioxidant capacities of aquatic organisms (Amado *et al,* 2009) and could be of great use in relation to *L. variegatus* following NP exposure. The absence of lipid peroxidation (as measured by TBARS) is similarly not fully representable of the potential damage incurred by *L. variegatus* following exposure. By introducing complimentary assays, investigating DNA damage (e.g. comet assay) or the formation of protein carbonyls, a superior understanding of potential oxidative damage could be inferred.

ANNEX A – Composition of OECD reconstituted water

250ml of solutions a-b were added to 10 L Milli-Q water following which, the medium was kept consistently aerated prior to use in testing.

(a) Calcium chloride solution
11.76g $CaCl_2$ x 2 H_2O in 1L distilled water.

(b) Magnesium sulphate solution
4.93g $MgSO_4$ x 7 H_2O in 1L Milli-Q water.

(c) Sodium bicarbonate solution
2.59g $NaHCO_3$ in 1L Milli-Q water.

(d) Potassium chloride solution
0.23g KCl in 1L Milli-Q water.

All chemicals sourced from Sigma-Aldrich® and of analytical grade.

ANNEX B – Composition of OECD formulated sediment.

Sediment constituent	Characteristics	% of dry sediment
Peat (Westland Garden Health Irish peat moss)	Sphagnum moss peat, air dried, particle size (≤0.5 mm)	2 ± 0.5
Quartz sand (Sigma-Aldrich)	Grain size: ≤ 2mm but > 50% of the particles in the range of 50-200 µm	76
Kaolinite clay (Sigma-Aldrich)	Kaolinite content ≥ 30%	22 ± 1
Nettle powder food source (Edinburgh Napiers)	Powdered *Folia urticae* leaves	0.4-0.5
Calcium carbonate	Chemically pure, pulverised $CaCO_3$	0.05-1
Deionised water	Conductivity ≤ 10 µS/cm	30-50

ANNEX C – Water parameters for sediment exposures

		pH				Temp (°C)				O_2 Content (%)		
Ag-NP concentration (mg/kg d.w. sed)	Wk 1	Wk 2	Wk 3	Wk 4	Wk 1	Wk 2	Wk 3	Wk 4	Wk 1	Wk 2	Wk 3	Wk 4
0	7.75	8.269	8.374	8.415	21.5	20.3	21.4	20.1	85.7	94.4	93.6	96.7
dispersant control	7.959	8.335	8.426	8.428	20.9	20.5	21	19.6	91.6	94.5	94.3	96.1
16.66	8.01	8.257	8.365	8.425	20.6	20.3	20.9	19.4	91.7	93.5	95.8	96
33.33	7.909	8.246	8.372	8.4	20.6	20.2	20.7	19.1	89.7	92.8	95.2	96
66.66	7.977	8.318	8.399	8.393	20.5	20.2	20.7	19.1	90	94.3	93.6	95.2
133.33	7.959	8.233	8.28	8.36	20.6	20.2	20.8	19.2	92.5	94.5	93.6	96

		pH				Temp (°C)				O_2 Content (%)		
TiO_2 Concentration (mg/kg d.w sed)	Wk 1	Wk 2	Wk 3	Wk 4	Wk 1	Wk 2	Wk 3	Wk 4	Wk 1	Wk 2	Wk 3	Wk 4
0	8.267	8.392	8.414	8.475	19.6	21.3	20.6	20.1	92.4	92.4	96.2	96.9
66.66	8.178	8.301	8.301	8.346	19.8	20	19.9	19.8	94.6	94.6	96.4	96.6
166.66	8.188	8.347	8.364	8.388	19.7	19.1	19.7	19.5	93.1	95.5	96.7	96.6
333.33	8.116	8.221	8.304	8.338	20	19.7	19.6	19.4	93.2	94.5	97.1	96.6
500	8.163	8.347	8.383	8.437	20.1	19.6	19.6	19.4	94.5	93.7	95.4	96.2
666.66	8.222	8.415	8.383	8.461	19.8	19.6	19.6	19.3	96.7	93.6	95.9	96

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